Identification of bacterial biothreat agents and pathogens by rapid molecular amplification methods

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Lecture Hall 1, Haartman Institute, Haartmaninkatu 3, Helsinki, on 12th May, 2017, at 12 noon

Helsinki 2017

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ISBN 978-951-51-3066-2 (paperback)
ISBN 978-951-51-3067-9 (PDF)

Unigrafia Oy
Helsinki 2017

To my loved ones, mom, dad and sis.

Tiedän jo mikä ovi pitää avata, vaikka se on piilossa

monien muiden sisään ja ulos johtavien ovien keskellä.

Tarvitsen vain hetken hiljaisuutta.

Paolo Coelho - Portobellon noita

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I **Matero P**, Pasanen T, Laukkanen R, Tissari P, Tarkka E, Vaara M, Skurnik M. Real-time multiplex PCR assay for detection of *Yersinia pestis* and *Yersinia pseudotuberculosis*. APMIS: acta pathologica, microbiologica, et immunologica Scandinavica 2009;117(1):34-44.

II Koskela, K A, **Matero P**, Blatny J M, Fykse E M, Olsen J S, Nuotio L O, Nikkari S. A multiplatform real-time polymerase chain reaction detection assay for *Vibrio cholerae*. Diagnostic microbiology and infectious disease 2009;65(3):339-344.

III **Matero P**, Hemmilä H, Tomaso H, Piiparinen H, Rantakokko-Jalava K, Nuotio L, Nikkari S. Rapid field detection assays for *Bacillus anthracis*, *Brucella* spp., *Francisella tularensis* and *Yersinia pestis*. Clinical Microbiology and Infection 2011;17(1):34-43.

IV Hirvonen J, **Matero P**, Siebert C, Kauppila J, Vuento R, Tuokko H, Boisset S. Novel portable platform for molecular detection of toxigenic *Clostridium difficile* in faeces: a diagnostic accuracy study. Eur J Clin Microbiol Infect Dis. 2016 Dec 17. (Epublication ahead of print).

ABBREVIATIONS

ab	antibody
ag	antigen
AG	The Australia Group
AHG	The Ad Hoc Group of States Parties that negotiated for a BWC verification protocol between 1995 and 2001
B	biological
BHI	brain heart infusion
BLAST	Basic Local Alignment Search Tool
BSL	biosafety level
BSA	bovine serum albumin
BT	biothreat
BW	biological warfare
BWA	biological warfare agent
BWC/BTWC	Biological and Toxin Weapons Convention
C	chemical
CBM	Confidence Building Measures
CCTA	cell culture cytotoxicity assay
CDC	US Centers for Disease Control and Prevention
CDI	*Clostridium difficile* infection
CDIW	*C. difficile* immune whey
CDT	*Clostridium difficile* transferase
CFU	colony forming unit
CHU	University Hospital Centre
CI	confidence interval
CLED	cysteine lactose electrolyte-deficient agar
Ct	Cycle threshold
CWA	chemical weapons agent
CWC	Chemical Weapons Convention
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay

ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ETEC	enterotoxigenic *Escherichia coli*
FMT	faecal microbiota transplantation
FH	field hygiene
FN	false negative result
FP	false positive result
FFP3	filtering facepiece, protection class 3
GDH	glutamate dehydrogenase
gDNA	genomic DNA
GHSA	Global Health Security Agenda
HEPA	High Efficiency Particulate Air
HUSLAB	Laboratory of the Hospital District of Helsinki and Uusimaa
IAC	internal amplification control
IAEA	International Atomic Energy Agency
IHR	International Health Regulations
iNAT	isothermal nucleic acid amplification technology
IPC	internal positive control
ISU	Implementation Support Unit (of BWC)
IVD	*in-vitro* diagnostic
JEE	Joint External Evaluation Process
LAMP	loop-mediated amplification technology
LB	Luria-Bertani agar
LF	lateral flow
LPS	lipopolysaccharide
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MSC	microbiological safety cabinet
NALF	nucleic acid lateral flow
NAT	nucleic acid amplification technology
NATO	North Atlantic Treaty Organization
NCBI	National Center of Biotechnology Information
NIAID	US National Institute of Allergy and Infectious Diseases
OCC	Operational Capability Concept
PaLoc	pathogenicity locus of *Clostridium difficile*
PCR	polymerase chain reaction

PFGE	pulsed field gel analysis
POC	point-of-care
PPE	personal protective equipment
RN	radiological/nuclear
RT	room temperature
rtPCR	real-time PCR
RPA	recombinase polymerase amplification
R&D	research and development
SC	United Nations Security Council
SEB	Staphylococcal enterotoxin B
SIBA	strand invasion based amplification
SIBCRA	sampling and identification of biological, chemical and radiological agents
spp.	species (plural)
TAT	turnaround time
TC	toxigenic culture
THL	National Institute of Health and Welfare
TN	true negative result
TP	true positive result
TS	tryptone soy agar
UK	United Kingdom
UN	United Nations
UNMOVIC	United Nations Monitoring, Verification and Inspection Commission
UNODA	United Nations Office for Disarmament Affairs
UNSCOM	United Nations Special Commission
US	United States of America
USSR	Union of Soviet Socialist Republics (former Soviet Union)
VBNC	viable but nonculturable (*V. cholerae*)
VEREX	Group of experts examining verification measures for BWC
WHO	World Health Organization
WMD	weapons of mass destruction
WWI	World War I
WWII	World War II

ABSTRACT

Background and aims

Rapid detection and identification of the pathogenic agents in biological weapons is critical in limiting their impact when used against civilian or military targets. Fast and accurate detection is also important in clinical microbiology where modern protocols seek to extend the diagnostic technology of automated central laboratories to the patient bedside or doctor's office, i.e., so-called point-of-care (POC) testing. The aim of this study was to develop rapid and accurate detection and identification assays for biothreat agents and other pathogenic bacteria from diverse sample types using molecular amplification methods. Secondly, the aim was to evaluate field-deployable platforms for use in remote or resource-limited locations.

Methods

This thesis consists of a series of studies of pathogenic bacteria that can cause severe disease in humans. The pathogens studied, *Yersinia pestis, Bacillus anthracis*, *Francisella tularensis*, *Vibrio cholerae,* and *Brucella* spp., have been listed as biothreat or biological weapons agents. In addition to the above-mentioned biothreat agents, real-time PCR assays were developed for the detection of *Bacillus thuringiensis* and *Yersinia pseudotuberculosis*. We also conducted an international multicentre accuracy study of a novel isothermal amplification platform for the detection of toxigenic *Clostridium difficile* from patient samples in the clinical context. The main methods used in these studies were real-time PCR and a novel isothermal amplification technique known as strand invasion based amplification (SIBA). Other methods used were bacterial culture, DNA isolation, conventional PCR, agarose gel electrophoresis, melting temperature analyses, cloning, DNA sequencing and different sample preparation methods. Several sample types, such as DNA, powder, spiked, environmental, and clinical samples were utilised in these studies.

Results

Multiplexed real-time PCR assay was developed for the simultaneous identification of *Y. pestis* and *Y. pseudotuberculosis*. The multiplex assay, which contained an internal amplification control, was shown to be specific and sensitive for the target microbes, and was validated with spiked clinical samples. For the detection of non-pathogenic and pathogenic *V. cholerae* from environmental samples, real-time PCR assays for the *toxR* and *ctxA* target genes were developed and specificity was tested with a panel of several serotypes of *Vibrio cholerae* and other bacteria and with spiked environmental water samples. The *V. cholerae* and real-time PCR assays for *Y. pestis*, *B. anthracis, F. tularensis*, and *Brucella* spp. were successfully tested with several real-time PCR thermal cyclers

and transferred to a small, field-deployable RAZOR instrument. A field-training assay with a simple sample preparation technique was developed and tested in field conditions for powder samples with insecticidal simulant containing spores of *B. thuringiensis*. In the final study, the accuracy of a newly-developed portable instrument, based on isothermal amplification, was assessed in an international multicentre study using 1160 faecal patient samples for the specific detection of toxigenic *Clostridium difficile.* This new test system was found to be comparable to the methods in routine use at three participating hospital laboratories.

Conclusions

Molecular amplification, such as real-time PCR and isothermal amplification are sensitive and specific methods that are suitable for rapid testing of bacterial biothreat agents and pathogenic bacteria from several types of samples following suitable sample preparation. With appropriate instruments, molecular nucleic acid amplification methods can be of significant advantage in field use for rapid identification of biothreat agents. These methods are also useful in POC situations where fast and reliable identification of pathogens is important. A robust and simple method of sample preparation represents the main obstacle to the development of user-friendly devices, especially for field use and POC applications.

1. INTRODUCTION

Whether naturally-occurring or intentionally released in a weaponized form, pathogenic bacteria can cause severe disease in humans. They can be used in acts of bioterrorism to disrupt civil society or in biological warfare against military targets. Throughout history, there have been many instances where biological agents have been used in a hostile way. One of the earliest incidents took place during the 14th century in the city of Kaffa, where Tatars catapulted human corpses infected with plague over the city walls (1). More recently, the threat of terrorist attacks using biological agents has increased (2). Due to the serious consequences of a bioterrorism, fast and accurate methods for the detection and identification of biothreat (BT) agents are needed. Effective implementation of public health measures and correct treatment of victims rely on accurate identification as well as for confirming that an attack with BT agents has taken place (3).

The World Health Organisation (WHO) defines a biological agent as one that produces its effect through multiplication within the target host and is intended for use in war to cause disease or death in humans, animals, or plants (4,5). Furthermore, some toxins produced by living organisms are classified as biological agents. A biological weapon (BW) includes both the biological agent and its delivery system. The Centers for Disease Control and Prevention (CDC) of the United States of America (US) defines a bioterrorism attack as the deliberate release of viruses, bacteria, or other germs (agents) for the purpose of causing illness or death in people, animals, or plants (6). It can also be stated simply that, use of bioweapons against the military is biowarfare, and when directed against civilian targets, it is bioterrorism (7).

BT/BW agents can be classified into categories according to their characteristics. CDC categorises agents into groups A, B, and C depending on importance. Category A agents include bacteria *Yersinia pestis, Bacillus anthracis*, and *Francisella tularensis*, as well as variola major, Ebola, Marburg, Lassa and Junin viruses and *Clostridium botulinum* toxin. These are considered to be priority agents because they can easily be disseminated or transmitted from person-to-person, cause high mortality with potential for major public health impact, cause public panic and social disruption, and require special action for public health preparedness. Category B agents such as *Brucella* spp. are moderately easy to disseminate and cause moderate morbidity or low mortality. Also food- and water-borne pathogens such as *Vibrio cholerae* are classified in this category. The third group, category C, includes emerging pathogens that could be engineered for mass dissemination in the future.

Several methods for the detection and identification of BT/BW agents have been developed. These include methods based on cultivation, biochemistry and immunology, nucleic acid amplification, mass spectrometry and bioluminescence (3) are in essence very similar to diagnostic techniques used in clinical microbiology laboratories. Emerging technologies, such as nanotechnology or whole genome sequencing, have promise in future clinical diagnostics

along with microfluidistics, isothermal amplification and smartphone applications (8–10). The most difficult task in the development of nano- or microfluidic diagnostic devices is a rapid and robust sample preparation protocol (11). Technology for BT/BW agent detection should be fast, sensitive, specific and preferably portable or field-deployable, and protocols should be simple enough to be performed by personnel who are not specifically trained for laboratory or medical work.

The development of rapid and accurate technologies for BT/BW agent detection could also benefit civilian POC diagnostics development, where the aim is to move from laboratory-based testing to the bedside of patients or to the doctor's office. In POC diagnostics, the aim is to diagnose the disease or cause of illness rapidly and accurately while the patient is being examined. The goal is to develop simple, sensitive and specific methods and robust devices for the rapid identification of the pathogen or biomolecular markers of the specific disease or condition. Available methods and many of the challenges facing POC device development are similar to those for BT/BW agent detection. Development of smaller, faster and more robust instruments as well as simple protocols that do not require extensive personnel training are target criteria for both applications. With these similar goals, the methods and devices developed could be used in both civilian and military applications and in clinical diagnostics and field detection of biological agents. Molecular amplification techniques have become standard practice in many biological fields over the past thirty years and in many cases have replaced the traditional laboratory methods for microbe identification.

In this work we have used molecular amplification methods to detect and identify bacterial BT/BW agents such as *Y. pestis*, *B. anthracis, F. tularensis, Brucella.* spp. and *V. cholerae.* We have developed amplification assays for these agents and a field-training assay for powder samples utilizing a non-pathogenic simulant agent, *B. thuringiensis*. We compared and evaluated molecular platforms with several polymerase chain reaction (PCR) assays for laboratory or field use with DNA, powder, spiked, environmental, and clinical samples. Finally, we conducted an international prospective accuracy study of a novel portable platform employing isothermal nucleic acid amplification technology for use in clinical settings for the detection of toxigenic *Clostridium difficile,* with altogether 1160 faecal patient samples.

2. REVIEW OF THE LITERATURE

2.1 Biological warfare and bioterrorism

Biological warfare has been used since Roman and Greek times (1). Early forms include projectiles (such as arrows and spears) coated with poisons or venoms, contamination of water and food supplies, as well as catapulting infected corpses over defensive structures and city walls during siege (7).

After chemical weapons were used on a large scale during World War I (WWI) to a horrific effect, the Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases and of Bacteriological Methods of Warfare was drafted in Geneva in 1925. Despite the Protocol, many states maintained research programs for developing biological weapons, since this was not explicitly prohibited (7). Bioweapon research peaked during World War II (WWII) (Table 1), and at least five nations have reportedly continued those programs (Table 2) or are suspected of doing so (7,12).

Table 1. Bioweapons research and development (R&D) by country after the Geneva Protocol was signed in 1925 and during WWII. Some of the R&D was for defence. Adapted and modified from Hilleman et al. 2002 (7).

Nation	Years R&D was conducted	No. workers during WWII	Focus during WWII
France	1922-1928, 1934-1940 (restricted)*	Unknown	Unknown
USSR / Russia	1926-1993*	Several hundred*	Typhus, plague
Japan	1932-1945	Several thousand*	Extensive: plague, typhoid, cholera, anthrax and others (limited information available)
Italy	1934-(restricted)**	Unknown	Unknown
Hungary	1936-unknown	Unknown	Unknown
UK	1936-unknown	40–50*	Unknown
Canada	1938-unknown	Small*	Animal and crop diseases, foot-and-mouth disease, anthrax
Germany	1940-(defensive only)*	100-200*	Offensive research forbidden
US	1943-1969	1,500–3,000*	Chemical herbicides, anthrax

* Estimated, **Did not engage in BW training or field testing

Table 2. Reported bioweapons programmes after WWII. Adapted and modified from Hilleman et al. 2002 and Leitenberg 2001 (7,12).

Country	Time	No. of personnel	No. of major facilities	No. of agents weaponised	Stockpiled	Used
UK	1945-1972	Unknown	1	0	No	No
US	1945-1969/1972	3,400	3	10	Yes	No
USSR/Russia	1945-1993*	Ca. 60,000	Ca. 35	10-12*	Yes	Unknown
Iraq	1975-2003	Ca. 300	7*	3**-5	Yes	No
South Africa	1981-1993	6-10	1-2	0	No	Yes

* Estimated, **According to UNMOVIC report (27), Iraq produced and weaponised three BW agents: *B. anthracis* spores, botulinum toxin and possibly aflatoxin. According to the Iraqi declaration, they also researched a number of other agents for BW purposes, including *Clostridium perfringens*, wheat cover smut, ricin, and trichothecene mycotoxins, camel pox virus, infectious haemorrhagic conjunctivitis virus (enterovirus 70) and rotavirus.

In the 1960s, President Nixon announced the dismantling of the US offensive biological weapons program. The Biological and Toxin Weapons Convention (in short BWC or BTWC) was drawn up to prohibit the development, production and stockpiling of bacteriological and toxin weapons. It was the first multilateral disarmament treaty to be signed and entered into force in 1975. As of December 2016, the convention has been signed by 182 States Parties, ratified by 178 States Parties and signed but not ratified by six signatory States (i.e., Central African Republic, Egypt, Haiti, Somalia, Syrian Arab Republic, and United Republic of Tanzania). At the time of writing, 12 nations (Chad, Comoros, Djibouti, Eritrea, Israel, Kiribati, Micronesia, Namibia, Niue, Samoa, South Sudan, Tuvalu) have neither signed nor ratified the convention (Figure 1) (13,14).

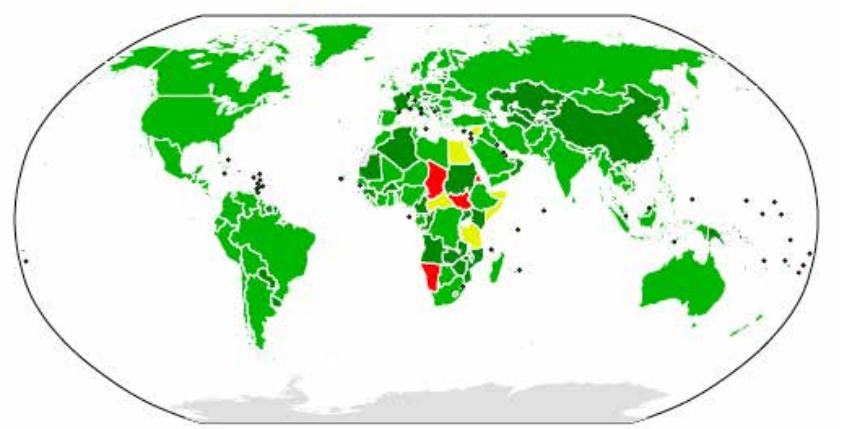

Figure 1. Countries in green have signed and ratified the treaty, countries in dark green have acceded or succeeded to the treaty, countries in yellow have signed, but not ratified and countries in red have not signed the treaty. Picture courtesy of Wikipedia By Allstar86 -CC BY-SA 3.0.

However, in the 1990s it became apparent that there was still a continuous threat of proliferation of BW agents by some countries, when anthrax spores were accidentally released from a bioweapons site in Sverdlovsk (Yekaterinburg) in 1979 (7,15). It is likely that during the incident, hundreds of people inhaled anthrax spores and many died in a zone downwind of the military facility. The exact number of casualties is unknown, but medical records indicate more than 100 fatalities. According to some references, livestock downwind of the facility also developed the disease (15). At the time, the Soviet government denied the incident and claimed that the deaths were due to contaminated meat or animal products. In the late 1990s, Soviet BW facilities were seemingly converted to civilian research institutes or employed in the production of antibiotics and vaccines. This was confirmed by the trilateral agreement among Russia, US, and UK and by President Yeltsin's decree of 1992 forbidding a Russian bioweapons program. However, there are indications that Russian BW capabilities still exist, in terms of remaining facilities, stockpiled and probably deployable BW agents that may be genetically modified, as well as expertise concerning their production (16).

Globalisation has enabled a more credible threat of bioterrorism and the recent escalation of terrorist attacks as well as efforts to acquire or develop BT/BW agents by terrorist groups. Aum Shinrikiyo, a religious cult in Japan, attempted to disseminate botulinum toxin and anthrax spores in Kameido but failed due to using an animal vaccine strain of *B. anthracis*, that lacks the pX02 plasmid and is thus not infective to humans (17). In 1994, they released sarin gas in the metro systems in Matsumoto with 7 deaths and 500 injured, and in Tokyo, where 12 died and more than 5500 people required medical care (18–20). In 2001, letters containing anthrax powder were sent in the US postal service to media and government representatives (21), highlighting the need for first responders develop a program for the rapid detection and identification of BT agents that can be used at the first contact location. In the US anthrax-letter case, 11 cases of cutaneous anthrax, 11 cases (five fatal) of inhalation anthrax, and approximately 10,000 were exposed to the spores and offered post-exposure prophylaxis (22,23). Since the 2001 incident, there have been thousands of mail hoaxes globally, many of them including a biological threat, e.g., bio-insecticide powders containing spores of *B. thuringiensis*, a close relative of *B. anthracis*, have been used in hoaxes in Australia (24).

2.2. Verification of the BWC and international efforts against biological and WMD threats

When it became clear that the BWC needed to be strengthened, a group of government experts was established at the Third Review Conference of the Convention in 1991. This working group was called VEREX and its role was to examine the potential verification measures that could be implemented by the States Parties from a scientific and technical point of view. Following this, in 1994, the States Parties established an Ad Hoc Group (AHG) that negotiated on a legally binding verification protocol between 1995 and 2001. The verification protocol included investigations of alleged use of biological weapons, site visits and declarations on activities in relevant biological fields. The negotiations were not successful, and were ceased at the Fifth Review Conference in 2001, when no agreement on the details of the verification measures could be reached, so the BWC still lacks effective measures to control compliance. In 2006, an agreement for establishing an Implementation Support Unit (ISU) to assist States Parties in implementing the Convention was reached, and the Confidence-Building Measures (CBM), requiring annual declarations from the States Parties concerning their activities relevant to the Convention that had been started in 1986, were re-enforced (14).

The most extensive and enduring international investigations into weapons of mass destruction (WMDs) are undoubtedly the United Nations' (UN) investigations of the Iraqi government led by Saddam Hussein. The United Nations Special Commission (UNSCOM) was established in 1991 by UN Security Council (SC) resolution 687 after the first Gulf War and the ceasefire agreement between Iraq and the coalition of States. Terms of the ceasefire and the

resolution called for the destruction of Iraq's WMD capabilities under international supervision. The UNSCOM was established to assist the International Atomic Energy Agency (IAEA) with respect to chemical and biological weapons. UNSCOM conducted over 250 field inspections between 1991 and 1998, during which it compiled information concerning Iraq's proscribed activities (Figure 2.). It was revealed by the inspections that the WMD programme in Iraq was more advanced than previously understood. Inspectors found hundreds of tons of chemical weapons agents (CWAs) and thousands of tons of precursor chemicals, BWA production facilities, missiles, launch pads, and missile warheads for both conventional and chemical munitions. Most of the agents, weapons and facilities discovered were destroyed. Iraq's BW programme was revealed by UNSCOM in 1995 despite denial and concealment by the Iraqi government (25). Many of the BWAs were destroyed by Iraq in 1991, but some remained unaccounted for (26). An export-import control of dual-use items, which can be used for both civilian as well as military purposes, was established for monitoring the sale of supplies and equipment to Iraq. Iraq was behaving uncooperatively with the inspectors, and accused them of espionage. After several years of trying to mislead inspectors, Iraq finally refused to cooperate with them altogether in 1998. The inspectors were withdrawn from Iraq before several days of air strikes against Iraq by the US and UK forces in an operation called Desert Fox.

Figure 2. UNSCOM inspectors at work in Iraq. Picture by UN Photo.

The following year in response to SC resolution 1284, a new commission referred to as the UN Monitoring, Verification and Inspection Commission (UNMOVIC) replaced UNSCOM. Its mandate was to disarm Iraq of its chemical and biological weapons as well as missiles with a range of more than 150 km. UNMOVIC trained an international group of experts as UN weapons inspectors, and they returned to Iraq in 2002 with the authority granted by SC resolution 1441 giving the UNMOVIC inspectors "immediate, unimpeded, unconditional, and

unrestricted" admission to any site in Iraq and the right to interview anyone they needed for their mission purpose. The UNMOVIC inspectors were headed by Dr. Hans Blix from Sweden as their Executive Chairman and worked intensively in Iraq between November 2002 and March 2003 for four months. During this time, UNMOVIC conducted a total of 731 inspections in 411 different sites and collected several hundred chemical and biological samples. Despite these efforts, no evidence of the continuation or resumption of WMD programmes or significant amounts of prohibited items was found. The UNMOVIC inspectors had to withdraw from Iraq in March 2003 before coalition forces led by the US resumed military activity in Iraq (27). The mandate of UNMOVIC expired in June 2007, and until that time it held several training courses for the experts on its roster and maintained an archive of the WMD inspections and materials recovered in Iraq.

The UN established The Office for Disarmament Affairs (UNODA) in 1998. UNODA is responsible for strengthening disarmament protocols with respect to WMDs including biological weapons. The Secretary General of The UN has a mechanism established in the late 1980s to carry out prompt investigations concerning the possible use of chemical and biological weapons. These investigations can be started at the request by any UN Member State, and the UN has a roster of experts and laboratories that can be employed in this purpose. As yet, the Secretary General's Mechanism has not been activated in response to the alleged use of BWAs, but it has been used in Thailand, Pakistan, Iran, Iraq, Mozambique, Azerbaijan and most recently in Syria (i.e., 2013) in order to investigate alleged use of CWAs (28).

In the health sector, the Global Health Security Agenda (GHSA) that was started by a US initiative in 2014 and involved over 50 countries and several organizations such as WHO and the European Union (EU), is strengthening the capability to address global threats to health and security. The agenda aims to build capacity in participating countries in order to recognize and respond to emerging threats. In Finland, the Ministries of Social Affairs and Health, Foreign Affairs, Defence, Interior, and Agriculture and Forestry are all participating in the programme. Finland was the chair of the GHSA programme in 2015, and is now acting as a member of the Steering Group and also the Chair of the Joint External Evaluation (JEE) Alliance until 2018 (29). JEE is a part of the GHSA and a voluntary and collaborative process to assess the capacity of a country to prevent, detect, and rapidly respond to public health threats of natural origin or due to deliberate or accidental release under the WHO International Health Regulations (IHR). The JEE assessment has been completed in 28 coutries so far; Finland's capability will be assessed in March 2017.

As part of the international effort opposing the development of chemical and biological weapons, an informal group of countries known as the Australia Group (AG) coordinates export control especially with regard to dual-use items. This helps countries fulfil their responsibilities under the BWC and CWC (Chemical Weapons Convention). The AG has 42 participants including the EU; Finland has been a member since 1991 (30).

2.3. Biothreat agents

Pathogenic microbes that can be used as BT or BW agents include bacteria, rickettsia and viruses, as well as toxins produced by microbes, animals or plants (4,31). These pathogens have the potential to cause high morbidity and mortality in humans, especially if they are dispersed in an aerosolised form. Most of these pathogens pose no major threat to public health under normal circumstances (32). Biological weapons include, in addition to the agent, their means of delivery through air, water, food, or via an animal vector (4,7). Biological agents have the capacity to cause epidemics and could be used in a hostile act by a State, or a State-sponsored or autonomous terrorist group. These pathogens can be found in nature, and could be modified in a well-equipped laboratory to become more lethal, infective, or resistant to medical treatment.

Potential biological agents have been listed by many organisations. Table 3 lists those agents relevant to BW and BT and the diseases they cause. One of the most well known and perhaps most cited list is the one provided by the CDC and which lists 32 potential BW agents classified in categories A to C (table 4). The AHG that negotiated a verification protocol for the BWC, also generated a list of biological agents of concern. It is often emphasised that these lists are not comprehensive but exemplary, since there are other agents that might be used, and new ones are emerging. Genetic engineering can be used to manipulate agents and modify their characteristics, such as virulence factors, in order to produce new and more lethal agents. Most of the lists include the classical BW agents, e.g., *B. anthracis*, *Y. pestis*, *F. tularensis*, *Brucella* spp., botulinum toxin, smallpox and viruses causing haemorrhagic fevers. *Bacillus anthracis*, *F. tularensis* and *Y. pestis* are considered category A BT agents in the CDC list due to the risk posed by their deliberate release. If inhaled, aerosolized spores of *B. anthracis* and *F. tularensis* can lead to inhalation anthrax and tularemia, respectively, but the diseases are not spread from person to person. In contrast, *Y. pestis* can cause pneumonic plague if the bacteria are inhaled and can spread from infected to healthy individuals (32).

Table 3. Biological organisms and toxins of relevance and/or the diseases they cause. Also, other agents and genetic modifications may have been researched. Adapted and modified from Hilleman et al. 2002 and Venkatesh et al. 2003 (7,4).

Bacteria (Including rickettsia and chlamydia)	*Bacillus anthracis (anthrax)* [a*,b, c]
	Francisella tularensis (tularemia) [a*,b]
	Brucella spp. *(brucellosis)* [a*,b, c]
	Yersinia pestis (plague) [b,c]
	Burkholderia mallei (glanders) [b]
	Burkholderia pseudomallei (melidiosis) [b]
	Coxiella burnetii (Q fever) [a*]
	Salmonella typhi (typhoid fever) [c]
	Shigella species (shigellosis)
	Vibrio cholerae (cholera)
	Rickettsia prowazekii (typhus fever)
	R. rickettsia (Rocky Mountain spotted fever)
	Chlamydia psittaci (psittacosis)
	Clostridium perfringens [c] (gas gangrene)
Viruses	Venezuelan equine encephalitis [a*,b]
	Yellow fever [a]
	Smallpox [b, c]
	Japanese encephalitis [b]
	Russian spring-summer encephalitis (Tick-borne encephalitis) [b]
	Ebola [b]
	Marburg [b]
	Lassa fever [b]
	Bolivian hemorrhagic fever (Machupo) [b]
	Argentinian hemorrhagic fever (Junin) [b]
	Camel pox [c]
	Rotavirus [c]
	Enterovirus 70 (Infectious hemorrhagic conjunctivitis) [c]
	Sin nombre
	Hantaan / Korean hemorrhagic fever
	Crimean-Congo hemorrhagic fever
	Rift Valley fever
	Dengue
	Omsk hemorrhagic fever
Toxins	Botulinum toxin [a*,c]
	Staphylococcal enterotoxin [a*]
	Ricin [c]
	Aflatoxin [c]
	Trachothecene mycotoxins [c]
Plant diseases	Rice blast [a]
	Wheat stem rust [a]
	Rye stem rust [a]
	Wheat rust [c]
Fungi	*Coccidioides immitis* (coccidioidomycosis)
	Histoplasma capsulatum (histoplasmosis)

[a]weaponised by the US, [a*]weaponised and stockpiled by the US, [b] researched, developed and some weaponised by the USSR,
[c]studied or declared by Iraq.

Table 4. The CDC risk categories for bioterrorism agents (6).

Category A*	Anthrax (*Bacillus anthracis*)
	Botulism (*Clostridium botulinum* toxin)
	Plague (*Yersinia pestis*)
	Smallpox (variola major)
	Tularemia (*Francisella tularensis*)
	Viral hemorrhagic fevers (filoviruses [e.g., Ebola, Marburg] and arenaviruses [e.g., Lassa, Machupo])
Category B**	Brucellosis (*Brucella* species)
	Epsilon toxin of *Clostridium perfringens*
	Food safety threats (e.g., *Salmonella* spp., *Escherichia coli* O157:H7, *Shigella*)
	Glanders (*Burkholderia mallei*)
	Melioidosis (*Burkholderia pseudomallei*)
	Psittacosis (*Chlamydia psittaci*)
	Q fever (*Coxiella burnetii*)
	Ricin toxin from *Ricinus communis* (castor beans)
	Staphylococcal enterotoxin B
	Typhus fever (*Rickettsia prowazekii*)
	Viral encephalitis (alphaviruses [e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis])
	Water safety threats (e.g., *Vibrio cholerae*, *Cryptosporidium parvum*)
Category C***	Emerging threat agents, eg.
	Nipah virus
	Hanta virus
	Tick borne encephalitis viruses
	Yellow fever virus
	Multidrug resistant *Mycobacterium tuberculosis*

*High-priority agents include organisms that pose a risk to national security because they can be easily disseminated or transmitted from person to person; result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption; and require special action for public health preparedness.

** moderately easy to disseminate; result in moderate morbidity rates and low mortality rates; and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance

*** These agents include emerging pathogens that could be engineered for mass spread in the future because they are easily available, easily produced and spread, have the potential for high morbidity and mortality rates and a major health impact. They are currently not believed to present a high BT risk to public health, but could do so in the future.

The following features can be used for categorising biological agents according to their potential use as BW or BT agents (4):

1. Infectivity – capacity to infect and multiply in the host
2. Virulence – severity of the disease caused
3. Lethality – ability to cause death
4. Pathogenicity – capacity to cause disease
5. Incubation period – time interval between exposure to the agent and onset of disease symptoms
6. Contagiousness – capacity to infect new hosts by direct or indirect transmission pathways

7. Stability – ability of the agent to survive in the environment
8. Ease of dissemination – how easy it is to disseminate the agent while maintaining the infectivity
9. Ease of production – how easily the agent can be produced in sufficient quantities
10. Available means of prophylaxis – availability of vaccines or prophylactic medication
11. Available means of cure – availability of therapeutic substances, such as antibiotics, antiviral agents, and antibodies

Table 5 lists the criteria and weighting the CDC used to evaluate potential biological threat agents.

Table 5. CDC criteria and weighting used to evaluate potential biological threat agents[a]. Adapted from Rotz et al. 2002 (33).

Disease	Public health impact		Dissemination potential				
	Disease	Death	P-D[b]	P-P[c]	Public perception	Special preparation[d]	Category
Smallpox	+	++	+	+++	+++	+++	A
Anthrax	++	+++	+++	0	+++	+++	A
Plague[e]	++	+++	++	++	++	+++	A
Botulism	++	+++	++	0	++	+++	A
Tularemia	++	++	++	0	+	+++	A
VHF[f]	++	+++	+	+	+++	++	A
VE[g]	++	+	+	0	++	++	B
Q Fever	+	+	++	0	+	++	B
Brucellosis	+	+	++	0	+	++	B
Glanders	++	+++	++	0	0	++	B
Melioidosis	+	+	++	0	0	++	B
Psittacosis	+	+	++	0	0	+	B
Ricin toxin	++	++	++	0	0	++	B
Typhus	+	+	++	0	0	+	B
Cholera[h]	+	+	++	+/-	+++	+	B
Shigellosis[h]	+	+	++	+	+	+	B

[a]Agents were ranked from highest threat (+++) to lowest (0).
[b]Potential for production and dissemination in quantities that would affect a large population, based on availability, biosafety level requirements, most effective route of infection, and environmental stability
[c]Person-to-person transmissibility
[d]Special public health preparedness needs such as stockpile requirements, enhanced surveillance or diagnostic need
[e]Pneumonic plague
[f]Viral hemorrhagic fevers due to Filoviruses *(*e.g., *Ebola, Marbur*g) or Arenaviruses (e.g., *Lassa, Machupo*)
[g]Viral encephalitis
[h]Examples of food- and water-borne diseases

2.4 Dissemination of biological agents

Terrorists could disperse biological agents by contaminating food or water sources or by using some sort of spraying or explosive device. Aerosol release of biological agents is considered to be the most likely route of dissemination, especially in the military context, but contamination of food or water may be simpler to achieve as it does not require sophisticated

delivery systems or necessitate a specific particle size for dispersal. Aerosol delivery of agents may lead to a more acute or severe forms of disease, as is the case with anthrax and pneumonic plague. The ideal size is around 5μm for a particle, which can be inhaled deep into the lungs of victims and dispersed over large areas by prevailing winds. Agents could be dispersed in wet or dry form and the range would be dependant on several factors, such as wind speed and direction, and characteristics of the agent such as the effect of ultraviolet light and desiccation. Several delivery systems (Table 6) have been developed, such as bombs, bomblets, and mines but these can be rather inefficient as the agents are often destroyed by the explosion (34).

Table 6. Delivery systems of BW agents by US bioweapons program between 1945 and 1969 and reportedly pursued by Iraq from 1975 to the first Gulf war of 1990-1991. Adapted and modified from Hilleman et al. 2002, and UNMOVIC 2003 (7, 27).

US	**Iraq**
Sergeant missile warhead	Missile warheads
Spray dispenser for drones	Aerial bombs
Wet and dry spray tanks for jet planes	Rockets
Cluster bombs	Dispenser / drone aircraft
Bomblet dispenser for long range bombers	Aircraft fuel drop tanks
	Helicopter spray
	Crop dusters

2.5 Characteristics of epidemics following a deliberate release

The outbreak of disease following a deliberate release of a biological agent may have several characteristics that should raise alarm and confirm the use of a biological weapon (35). Firstly, there may be an unusually large number of people infected with a disease that is not endemic to the region, or a sudden outbreak of a zoonotic disease among livestock with no natural explanation. Secondly, morbidity and mortality may suddenly spike. This may be due to higher virulence and pathogenicity caused by genetic modification or exposure to a higher density of agents than would occur under normal circumstances. Furthermore, the disease characteristics may be unusual, e.g., 95% of anthrax cases worldwide are cutaneous, so a sudden increase in the incidence of inhalation anthrax would be unusual. Thirdly, the disease may be uncommon to the area or not have occurred there previously. The source of the outbreak may radiate from a centre point (a one-point outbreak) or the infection may present a line of dissemination as might be created by a light aircraft fitted with a crop sprayer. In 1970, WHO estimated the number of casualties following an aerosol attack of a city of 500,000 (Table 7). One might expect to see a spike in the number of cases and a short incubation period due to the large inoculum. There may also be multiple epidemics of the same disease occurring simultaneously in several sites, as would be the case when dissemination of an agent took place at multiple locations simultaneously (35).

Table 7. WHO estimate of casualties after an aerosol attack of 50 kg of a BW agent, a downwind delivery path of 2 km, in a city with a population base of 500,000 (e.g., Helsinki). Adopted and modified from Bellamy and Freedman 2001 (36), original data reported by a WHO consultant modelling the attack on Geneva in 1970 (5).

Agent	**Wind downside reach (km)**	**Number killed**	**Number incapacitated**
Rift Valley fever virus	1	400	35,000
Tick-borne encephalitis virus	1	9500	35,000
R. prowazekii (typhys)	5	19,000	85,000
Brucella spp. (brucellosis)	10	500	125,000
C. burnetii (Q fever)	>20	150	125,000
F. tularensis (tularemia)	>20	30,000	125,000
B. anthracis (anthrax)	>20	95,000	125,000

2.6 Defences against biological agents

It is difficult and expensive to provide constant protection for a large population facing diverse and dynamic biological threats. Vaccination against agents can offer some prophylaxis if the vaccine is available in sufficiently large quantities. Vaccines may not be effective against naturally-mutated or genetically-modified pathogens. This is certainly the case for the Influenza virus that mutates naturally so fast that a new vaccine must be developed almost every year. In Russia, vaccines against many BT/BW agents have been developed and new vaccines are currently being modified and improved (37). Antibiotics and antivirals can be used as prophylaxis in many cases, but they are ineffective against certain pathogens. Furthermore, due to their over-use in animal production and human medicine, the abundance of antibiotics in the natural environment has led to a growing concern among microbiologists and public health personnel that natural selection of microbial resistance could soon mean we have few left to treat increasingly stubborn infections (38–40).

Mechanical defence against BT/BW agents can be in the form of a sealed shelter constructed of a non-permeable material and supplied with filtered air. Respiratory equipment with appropriate filters and other personal protective equipment (PPE) that prevent infections through broken skin or wounds are also recommended. Traditional disinfectants, such as formaldehyde and chlorine solutions, are useful for surface decontamination (41), but some BT/BW agents, such as bacterial spores, can persist on surfaces and in the environment for prolonged periods of time and protocols should account for this potential source of reinfection (42,43).

2.7 Biological agent detection methods

2.7.1 Traditional methods in microbiology

Until recently, clinical microbiology relied solely on diagnostic methods such as plate culture and phenotypic, biochemical, and serological tests for the identification of human pathogens (32,44). Classical identification methods can be very sensitive and reliable, but they require dedicated laboratories, highly-skilled personnel, and can take from days to even weeks to perform. Furthermore, cultivation of dangerous pathogens also involves extensive biosafety measures (32). As such, classical methods are inappropriate for field use and they cannot provide the identification in real-time. An ideal detection system would be sensitive and specific, easy to use, portable, and provide accurate results in minutes or hours in order to reduce the impact of a bioterrorism event (32,45). It is important to identify the BT/BW agent as soon as possible so that agent-appropriate measures can be taken and any spread of the disease is minimized (32).

In spite of these issues, traditional methods have been used to identify BT/BW agents. This is partly due to variability and complexity of samples to be analysed and some of the pathogens involved. In a bioterrorism event, samples can be composed of air, soil, surface swabs, tissues and fluids from animals and humans, or even the agent itself. Culturing biological agents, especially viruses, can be difficult or even impossible (46). Even when practical, viral culture can take days to weeks before a decisive result is obtained whereas molecular assay can identify an unknown pathogen in a matter of hours (44). This is the one of the main reasons why clinical virology has largely shifted to molecular testing with reduced turn-around time (TAT) and increased sensitivity and specificity.

In addition to laboratory culture, automated biochemical tests have been used to detect and identify BT/BW agents. Automated identification test systems that are used widely in clinical microbiology laboratories are based on metabolized substrates or susceptibility to antimicrobial chemicals. Biochemical test strips, such as the API series (3) and gas chromatography have been used to identify *B. anthracis* spores (47), F*rancisella* spp. (48,49) and *Yersinia* spp. (50).

2.7.2. Immunoassays

Immunoassays have been used extensively to detect infectious disease agents, bacteria, viruses, spores, and toxins. Classical immunoassays are enzyme-linked immunosorbent assays (ELISA) (51). The immunological applications are based on a specific reaction between an antibody (ab) and antigen (ag). The primary antibody (specific to the detected ag) is normally conjugated to a marker. In particle-based applications, these markers are usually gold or fluorescent particles. A secondary antibody (specific to the primary-ab) then captures the ab-ag complex leading to formation of a detectable colour or fluorescence. The success of immunological tests depends on the formation of the ab-ag complex and the ability to detect

it. Specificity is limited by the quality of the antibody used, and sensitivity is typically less than that of DNA-based assays. Detection limits of immunoassays have typically been around 10^5 colony forming units (CFUs) per test (3), but modern systems have lowered this to 10^4 CFU/ml or 100 CFU/test (52). Immunoassays can be performed on several types of platforms; those most often used in connection with BT/BW agent detection include lateral flow (LF) strips based on immunochromatography. These devices are also referred to as hand-held assay devices, or smart tickets (Figure 3) (3).

Figure 3. Example of lateral flow test strips used in the field to detect and provide preliminary identification of BT/BW agents. The test result is obtained within 10 to 15 minutes. Test strips are easy to use and transport.

LF assays have been developed for *B. anthracis* (53), *F. tularensis*, *Y. pestis*, botulinum toxin (54), *Brucella* spp., ricin toxin, and Staphylococcal enterotoxin B (SEB) (52,55–58). The benefits of LF tests are rapid results, low cost and simplicity. These devices are small and easy to transport and store. LF assays are also easy to perform and the result is normally obtained within 10 to 15 minutes. The sensitivity of an LF test is between 60% and 95%, and the visual interpretation of results is subjective and can lead to false-positives or false-negatives. This drawback can be overcome by using readers that eliminate interpreter subjectivity (59). LF tests can be less sensitive and sometimes less specific in comparison to nucleic acid amplification technology (NAT) based assays, and sometimes show cross reactivity (60). However, recent developments, for example on surface-enhanced Raman-scattering –based LF sensors have significantly improved the sensitivity of LF tests (61).

2.7.3. Molecular amplification methods

Diagnostic methods used for naturally-occuring microbial diseases also work for standard BT and BW agents. However, genetic modification could enhance their virulence, resistance to antibiotics and vaccines, or other characteristics (62,63). Agents could also be engineered to be easier to handle, more stable in the environment or more difficult to detect by

conventional methods. In response, molecular technologies can be developed to detect emerging and genetically-modified agents, e.g., primers in PCR-based assays can be redesigned and sequencing can provide genomic information concerning the agents involved (62).

Amplification of nucleic acids using PCR was invented in the 1980s by Kary Mullis and co-workers and has been widely used ever since in research laboratories and remains the main tool in modern molecular diagnostics (44,64). PCR has many desirable properties, such as high sensitivity and specificity. Buchan and Ledeboer have stated that NATs have been shown to be more sensitive than culture methods for pathogens in clinical samples, since the pathogens may lose their viability during sample transport. The detection limit can be 1-10 copies of isolated genomic DNA per PCR reaction. NATs have greatly reduced the time needed to detect and identify microbes (44). With real-time PCR (rtPCR) using fluorescent probes and detectors, results can be obtained within a few hours including sample preparation (65,66). In the case of a suspected event involving BT/BW agents, unknown samples must be handled in biosafety level 3 (BSL-3) or 4 (BSL-4) conditions. Following pre-treatment, the isolated genetical material can be transferred to and amplified in a standard molecular biology laboratory. The drawback of NAT testing is that a positive result provides no information concerning the viability of any microbes present, only that their DNA or RNA is present in the sample (32).

2.7.3.1 Multiplexing of targets in molecular amplification

In molecular amplification methods, such as PCR or isothermal amplification, several target areas can be combined to one amplification reaction in so-called multiplexed detection. Up to 20 different targets have been combined in a single panel (67,68). For example, multiplexed diagnostic panels exist for suspected respiratory or gastroenterological infections, for sepsis and positive blood cultures, and for biothreat agents (32,69–71). Multiplexing is an attractive diagnostic technology as it reduces the required sample volume and handling time, lowers TAT, cost and the risk of contamination. In addition, multiplex panels simplify the ordering of tests by the clinician. However, test results must be interpreted cautiously since the presence of a nucleic acid does not always correlate with clinical illness, and asymptomatic carriage must also be taken into consideration. For example, up to 10% of stool samples may be positive for multiple targets, which can be an indication of co-infection or asymptomatic carriage. Positive results may also be caused by residual nucleic acid in the absence of viable organisms following treatment (72,73). Multiplexing large panels in one assay will also affect the sensitivity of the individual assays, which may limit the use of highly multiplexed tests for diagnostics of severe infections (74). Also, it may in some cases be less cost-effective than testing for single pathogen targets (67).

In many commercial assays, internal amplification controls (IACs) are used to detect and control for false-negatives. Multiple gene targets for each organism can also be used, which will reduce the chance that strain variants are missed due to mismatched primers.

Amplification of multiple target sequences per organism also reduces false-positive or false-negative results. Amplification inhibitors or pathogens with similar sequences may be present, especially in complex samples such as soil and other environmental samples. Therefore, it is essential to thoroughly test any newly-designed PCR assays with multiple species and strains of target and closely-related microbes in order to verify the analytical specificity of the assay (32).

2.8 Analysing biothreat agents in the field

2.8.1 Sampling BT/BW agents

Sampling is one the most critical steps in the efficient detection of BT/BW agents. The samples must be taken at the right time, in the right place and in an appropriate quantity and density. If the samples are not collected correctly and with appropriate equipment, or they are transported in unsuitable media or conditions, tests results cannot be relied upon. Therefore, it is important that a sound sampling strategy is in place and samples are collected by properly-trained personnel equipped appropriately and employing all safety and quality-control measures (75). The sampling strategy depends on the specific information sought. When collecting biological samples, the use of sterile equipment and aseptic technique is essential. In the case of a suspected deliberate release of BT/BW agents, samples should be taken with forensic techniques and maintaining the chain of custody with rigorous documentation that can be presented to an international court as evidence of their use. The location, number and quantity of samples to be taken should be planned carefully and agreed with the laboratory that will receive and process them. Samples can include fluids, soil, vegetation, surface swabs, munition fragments, as well as the pure agents themselves.

A team trained in CBRN (chemical, biological, radiological and nuclear) sampling is usually composed of a team leader, a sampler ("dirtyman") and a sampling assistant ("cleanman"). In addition, there may be other support personnel in a team with responsibility for communications and security. All team members entering the site suspected to be contaminated (the "Hot Zone") must use appropriate PPE and must be thoroughly decontaminated upon leaving the area. Also, all equipment that the sampling team removes from the Hot Zone must be decontaminated to avoid the spread of any agents. Therefore, only the equipment necessary for sampling should be taken inside or removed from the site.

2.8.2 Mobile laboratories

Mobile facilities have been developed for use in situations where laboratory services are poor or otherwise unavailable, such as Africa. For example, WHO has deployed mobile field laboratories in response to the outbreak of Ebola in West Africa in 2014-2015 through its Emerging and Dangerous Pathogens Laboratory Network (EDPLN) and Global Outbreak Alert and Response Network (GOARN). A total of 32 mobile laboratories were deployed in Nigeria,

Sierra Leone, Mali, Cote d'Ivoire and other countries in West Africa during the Ebola outbreak. Mobile laboratories were deployed from the US, UK, Canada, China, South Africa, Italy and the Netherlands. All of these laboratories had different set ups but most relied on rtPCR as the primary detection method (76). These laboratories were set up in temporary buildings in remote areas or in permanent buildings in cities or towns near to or in Ebola treatment facilities. The rapid deployment of these laboratories increased the diagnostic capability and improved preparedness in the countries directly affected by Ebola and their neighbours (77). A German facility was deployed in Guinea within the European mobile laboratory project in West Africa as a part of the WHO response (78). This modular facility was packed into cases that can be easily transported to and modified at the site to suit the mission. Key elements in a successful mobile laboratory include expert personnel, stringent biosafety and biosecurity measures, appropriate methods and equipment, as well as adequate logistical support for the team and the laboratory in the field. Mobile laboratories can be used for field operations in natural disease outbreaks (e.g., Ebola in West Africa) as well as suspected BT/BW attacks or field investigations and inspections, where they provide detection and identification capabilities at a site, when fixed-location laboratories are difficult to reach or their capacity is insufficient.

2.8.2 The Finnish Mobile CBRN Laboratory

The Finnish Mobile Diagnostic CBRN Laboratory (Field Laboratory) has been developed for military use in a variety of defence operations and during natural or man-made disease outbreaks and incidents. The field laboratory can be used for national defence tasks, international crisis management and to support other Finnish authorities. The laboratory has been evaluated and approved by the North Atlantic Treaty Organization (NATO) as the first mobile CBRN laboratory in the Operational Capability Concept (OCC) program, and is internationally well recognised. The Deployable CBRN Laboratory includes a command element and three platoons: HQ and Logistics Platoon; CBRN Platoon that is responsible for reconnaissance and decontamination; and the CBRN field laboratory platoon. The latter includes the field laboratory and a SIBCRA (sampling and identification of biological, chemical and radiological agents) patrol that is specialised in forensic CBRN sampling (79).

The field laboratory is divided into chemical (C) and biological (B) sides of a converted semi-trailer, as well as a radiological/nuclear (RN) and field hygiene (FH) laboratories in adjoining tents (Figure 4). The biological side is equipped as a BSL-3 laboratory with negative air pressure and high efficiency particulate air (HEPA) filtration. The personnel put on PPE in an anteroom tent before entering the B-laboratory. Inside the B-laboratory, samples are processed in microbiological safety cabinet (MSC) III and an adjoining MSC-II cabinet. Analytical instruments are located on the bench top and laboratory waste is autoclaved inside the laboratory. The entire laboratory can be decontaminated with a H_2O_2 vapour system and MSCs can also be decontaminated separately. All laboratory equipment can be packed into boxes for transport by land, sea or air (79).

Field laboratory personnel are well trained in their respective fields. The laboratory has a leader and two scientists in each of the B- and C- laboratories (Figure 5). One scientist is responsible for the RN laboratory and another for FH. All personnel produce risk assessments for the area of operation and their work in the laboratory. They work closely with the SIBCRA patrol, composed of a team leader and three specialists (C, B, and RN, respectively) that have received an advanced scientific education and have been trained in SIBCRA sampling protocol according to NATO standards.

Figure 4. The Finnish Mobile CBRN Laboratory. Upper left: schematic rendering of the field laboratory and adjoining tents that house the FH and the RN capabilities. Upper right: the field laboratory land transport. Below: the field laboratory air transport. Photos reproduced with permission of the Finnish Defence Forces.

Figure 5. Upper left: rendering of the field laboratory semi-trailer. Upper right: member of the SIBCRA sampling team taking a sample during a field exercise. Below left: Scientific personnel in PPE inside the B-laboratory (BSL-3) Below right: Scientific personnel at work in the C-laboratory. Photos reproduced with permission of the Finnish Defence Forces.

2.9 Point of care (POC) in diagnostic microbiology

2.9.1 Diagnostic microbiology

In clinical diagnostic microbiology, patient samples are screened for the pathogen typically associated with a given disease or infection. Infectious diseases are the primary cause of mortality worldwide and claim about one quarter (i.e., 14 million) of the approximately 56 million deaths that occur each year (11). Because of the life-threatening nature of infectious agents, rapid identification is required for the timely application of treatment and isolation of infected patients in the case of highly contagious diseases (80). Identification of the microbe causing the infection, and determination of its antimicrobial susceptibility or toxin production profile, is vital information for decision makers during the first few critical hours of patient management (81). Antimicrobial drug resistance and toxin production profiles are often

assessed to determine the correct treatment for each patient. Healthcare systems are responsible for providing high quality diagnostic services that enable the evidence-based treatment of patients, and prevent the misdiagnosis of patient illness and treatment failures (82).

As mentioned earlier, culture-based methods were the gold standard in microbial diagnostics and in regular use until the late 1990s. Culturing requires viable infectious agents to be present in the sample, which means that transport and storage conditions of the sample play an important role in the diagnostic protocol (83). This is one of the main reasons why NAT-based tests have become more commonplace in clinical microbiology diagnostics since the early 2000s; they do not require live microbes and sample transport conditions are less critical (84,85). NAT tests also offer high sensitivity and specificity as well as shorter TAT with improved detection of target organisms when compared to culture methods (86,87).

Recently, many clinical labs have begun relying on large centralized facilities with automated high-throughput instruments but at the same time, nucleic acid amplification based point-of-care (POC) diagnostics has become more popular. POC has been developed for more decentralized settings by simplification of the testing process and miniaturisation of testing platforms (80,88,89). In 2010, Bissonnette and colleagues stated that diagnostic microbiology and patient health would benefit most from microbial diagnostics that is at least as fast as biochemical testing, but with improved sensitivity. Until recently, rapid LF tests were the only type of POC tests available (90).

2.9.2 Definition and criteria of POC testing

POC testing has been defined by Ehrmeyer and Laessig (91) as patient samples being analysed near or at the patient, and the test results being available instantly or in a very short time frame. A more simple definition of a POC test is that it is performed at the site of patient care (92,93). There are two categories of technology that can be used for POC testing; first, small bench-top analysers and second, hand-held single-use devices (92). According to Price, advantages of POC testing include faster decision making, earlier treatment, improved adherence to treatment, reduced incidence of complications, faster treatment optimisation, lower re-operation or re-admission rate, and better patient satisfaction (92).

Performing POC tests should not require significant laboratory infrastructure or highly-skilled personnel. POC tests are designed to be easy to use and interpret, and give fast results (94). Another key advantage of these new diagnostic tests is that they are potentially cheaper than conventional tests (59). According to Drancourt et al., POC tests can be used in clinical diagnostic testing of remote or isolated populations and deliver results more rapidly and thereby enhance disease management and patient treatment. Fournier et al. and Drancourt et al. have also stated that diagnostic microbiology has changed radically during the 21st

century, and today it is possible to obtain a diagnosis during patient evaluation using the latest molecular techniques with lower production costs (59,95).

POC testing has entered clinical practice in developed and developing countries in applications that require fast TATs and in settings where reliance on a centralized laboratory is not practical or possible (96). In most developing countries, laboratory services are limited to large cities and high-quality diagnostic tests are not available to many patients (82). Although automated on-demand or single-test formats are often more expensive than batched testing, Buchan et al. stated that the total cost of patient care could be reduced by rapid diagnostic results if the price is calculated per test (44). Many lives could be saved by POC tests in developing countries if they became routinely applied for diagnostics and thereby making treatment programs more efficient (11). Mabey et al. recommended that developers should take into account the ASSURED criteria (Table 8), which define the ideal characteristics of a POC test (97). Many of these requirements are similar to those of rapid detection devices for BT/BW agents.

TABLE 8. The ASSURED criteria of an ideal rapid test, adapted form Mabey et al. 2004 (98):

A	=	Affordable
S	=	Sensitive
S	=	Specific
U	=	User-friendly (simple to perform in a few steps with minimal training)
R	=	Robust and rapid (can be stored at room temperature and results available in <30 min)
E	=	Equipment-free or minimal equipment that can be solar-powered
D	=	Deliverable to those who need them

POC technology seeks to enable diagnosis to be performed closer to the patient (99,100). Bissonnette et al. have concluded that POC testing has the potential to provide diagnostic results within the optimal timeframe for infectious disease management and that "molecular diagnostics provide the best platform for impacting on human health in developed countries". (11). Recent studies also suggest that cost-effectiveness of POC tests for diagnosing infectious diseases, such as HIV or malaria, support their use in resource-limited settings. Standard analyses do not take into consideration the reduction in patient waiting time and number of required visits or the benefits of correct medication and treatment that rapid POC testing provides (100). Rapid diagnosis of life-threatening infections such as sepsis is equally important in more developed countries. Tissari et al. evaluated a new DNA-based microarray platform to diagnose sepsis and concluded that it was highly sensitive and specific to the bacteria involved and saved time compared to culture-based methods. The assay would enable fast treatment decisions for critically ill patients thus improving patient prognosis and reduce the costs associated with disease management (101).

It must be noted that although POC diagnostics clearly have many benefits, there may be problems with the quality of results, when testing is moved outside laboratories and is performed by non-specialists. While manufacturers make every effort to develop tests that

are robust and easy to use, and are required to evaluate the tests with the intended users, there is always the possibility of operator error. The human factor must be taken into consideration already in the design phase of POC tests. Qualified laboratory personnel should control the purchasing and evaluation process of POC instruments for hospital laboratories. Specific scorecards have also been developed to help laboratories and health authorities to evaluate and select appropriate diagnostic devices for use based on the operational specifications of the tests Specific scorecards have also been developed to help laboratories and health authorities to evaluate and select appropriate diagnostic devices for use based on the operational specifications of the tests (102). The quality of POC diagnostics should be carefully evaluated before putting into use, and thorough and continuous training must be provided to those conducting and interpreting the test (103). POC-test manufacturers should make results simple and objective to interpret. It is still advisable to have a laboratory backup in advancing on the proper use of POC tests and on the interpretation of the results. Continued training and technological updates on diagnostics should be provided to all medical personnel and healthcare providers. When POC testing is rigorously evaluated, correctly used, and effectively regulated, it can be used to improve health both in developed and developing countries (82).

With these preconditions, POC testing may benefit the healthcare system by reducing overall costs and by providing evidence-based information for timely and correct treatment of patients. When using POC testing, especially in remote areas, it is necessary to have a reach back support of clinician specialised in infectious diseases, who can make informed treatment decisions of patients based on solid diagnostic evidence.

2.9.3. NAT POC and isothermal amplification

Many of the modern molecular systems for clinical diagnostic applications have been relatively complex, expensive and the most of them are based on PCR and real-time fluorescence detection. NAT-based technologies developed for POC applications have improved rapidly during the last ten years and are now in use throughout the world (104–106). Cepheid was perhaps the first company that successfully demonstrated the possibility of using NAT in POC, and the GeneXpert platform was first developed to detect BT/BW agents and then successfully moved into diagnostic applications (96).

Lately, isothermal nucleic acid amplification technologies (iNATs) have been used in diagnostic applications. Some iNATs offer the same sensitivity as molecular amplification techniques but with a simpler device or instrument platform than rtPCR. In iNATs, amplification is performed at a constant and lower temperature so there is no requirement for expensive thermo cyclers although they can be used for isothermal applications as well. According to Niemtz et al., if a more simple sample preparation and instrument technology can be used with iNATs, the workflow can be made easier and more suitable for use in low-resource settings and closer to the patient (96).

Some of the iNATs provide results in 15 minutes or less via a battery-powered instrument. For example, strand invasion based amplification (SIBA) (Figure 6) or recombinase polymerase amplification (RPA) technology can be used for rapid detection of pathogens (104,107–111). Detection technologies can be simplified to overcome the need for sophisticated optics, which further reduces the instrument cost and would hasten the development of low-cost molecular POC devices. According to Buchan et al., these technologies can improve patient care and reduce TAT and the overall cost of healthcare (44).

2.9.4 Integrated NAT POC devices

Before the result can be obtained on a NAT device, three main steps must be completed; the preparation of the sample, amplification of the desired target, and detection of the amplified product. The last two steps are usually solved by test developers, but Niemz et al. who reviewed NAT POC technologies for infectious agents concluded that the integration of sample preparation with amplification and detection in a cost-effective, robust, and user-friendly format is the main challenge for test developers (96). Recently, PCR assays have become less prone to inhibition (112,113) and some isothermal technologies are inherently rather robust, and could require less rigorous sample preparation (114). According to Niemtz and colleagues "Isothermal amplification methods often require less-stringent sample preparation, and can facilitate rapid, sensitive, and specific target amplification via single-temperature incubation, which reduces system complexity and cost compared to PCR-based methods" (96). They also proposed that combining iNATs with endpoint detection, e.g., visual methods such as nucleic acid lateral flow (NALF), would be a good way of lowering the cost of POC devices while at the same time providing the required sensitivity. When using eg. NALF, instrument may not be needed. POC NAT tests and methods first developed for non-clinical applications such as biothreat agent detection, food-safety, or veterinary diagnostics can serve as stepping stones towards clinical diagnostics (96), which is in most countries more highly regulated and therefore more costly and time consuming to develop.

Many advances in NAT diagnostic technologies and miniaturization of devices have been made in the past 20 years, and several commercial platforms are now on the market. According to Buchan and Ledeboer, these platforms are currently best suited to laboratory use but the the goal is to use them in POC tests as well. This can be achieved through simplification of the testing process. Some of the available platforms lack automation for sample processing but have been simplified and could prove useful in POC or "near-POC" diagnostics (44). For example, a newly developed platform based on isothermal SIBA technology (Orion GenRead) is a small, portable, battery operated and rather simple "near-POC" format (115). Nanobiosensors that can detect small amounts of microbes from biological samples in seconds, and hand-held integrated macro- or microfluidic so-called lab-on-a-chip systems have recently been developed for multiplex detection of infectious diseases (106,116–124).

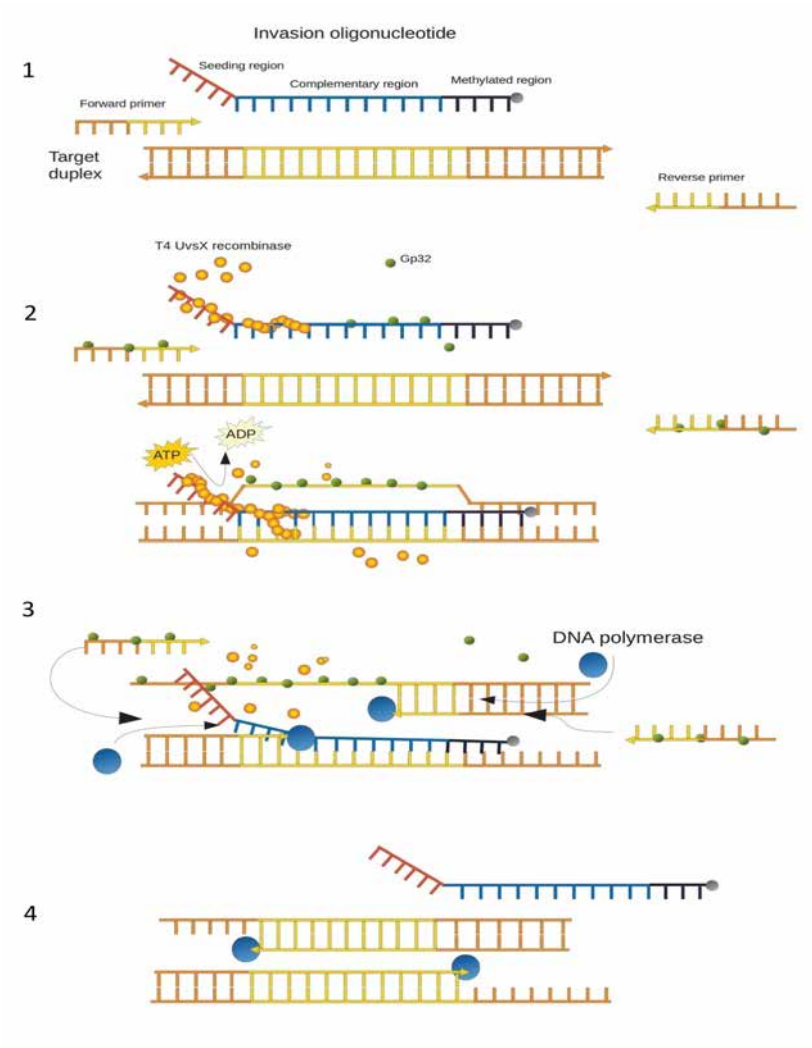

Figure 6. Principles of the SIBA reaction. 1. The SIBA reaction requires target-specific forward and reverse primers and an invasion oligonucleotide (IO) shown at the top of the picture. The IO has three main parts: the seeding region, the region complementary to the target sequence, and the methylated region. 2. The single-stranded elements are coated with gp32 protein that stabilises them. T4 UvsX recombinase polymerizes to the IO thereby displacing the bound gp32 while the primers are too short for UvsX binding. The IO then invades the region with complementary sequence in the target DNA through the activity of UvsX. The invasion process facilitates the separation of the target duplex, allowing target-specific primers to bind. ATP is used as an energy source during binding and dissociation. 3. Polymerase capable of strand displacement then extends the DNA strand from each primer. 4. This event leads to the production of two copies of the target and the process continues amplifying the target region exponentially. The reaction is conducted in a constant temperature around 40 °C. Adapted and modified from Eboigbodin et al. (111), figure by Tuomas Ojalehto.

2.10 Select bacterial pathogens used in this research

2.10.1 Yersinia pestis

Yersinia pestis is a Gram-negative enterobacterium. It is the causative agent of bubonic, pneumonic, and septicemic plague, one of the deadliest bacterial pathogens affecting humans. During the Middle Ages, it was responsible for the Black Death in Europe killing 30-50% of the European population at the time (125,126). It has been classified as a BW/BT agent due to its potential lethality (127) and has caused three major pandemics: Justinian's plague from 6th to 8th centuries; the Black Death of the 14th century; and modern plague since the 19th century (128). The first pandemic started in Africa and spread to the Mediterranean, the second, the Black Death, originated from Central Asia and spread to Europe from the Caspian Sea, and the third pandemic began in Southwest China and spread globally via ships from Hong Kong (129). The pneumonic and septic forms of plague are almost always lethal if the patient does not receive proper treatment. Millions of people have died in plague epidemics throughout Europe, Asia and Africa. Plague is spread by fleas and rodents, which are the natural animal reservoirs of the bacterium. *Yersinia pestis* is endemic to parts of Africa, Asia and America and WHO reports 1000-3000 worldwide cases of plague each year (31,128). It has been estimated that 200 million people have died of plague during human history (128).

Nowadays, most cases of plague occur in rural communities as better sanitation and antibiotics have reduced the epidemics in urban settings (31) *Yersinia pestis* can be effectively treated with antibiotics, although resistant strains have been detected (130,131). Plague has been classified as a re-emerging disease since the 1990s and there have been cases of infection in areas where plague had previously been absent for decades, including the Mediterranean region, Jordan, Algeria and Libya (132). Madagascar has been an important focus of plague since 1991, when a cluster of cases occurred after 62 years without the disease (133). Between 2000 and 2010, approximately 7,000 cases were reported in Madagascar, but the Democratic Republic of the Congo was the leading country with over 10,500 cases (134). According to WHO, the annual number of cases reported worldwide between 2010 and 2015 ranged from 320 to 772 (135).

2.10.1.1 Subtypes of Y. pestis

Yersinia pestis is closely related to the enteric pathogen *Yersinia pseudotuberculosis*. It has been estimated that the divergence of *Y. pestis* from *Y. pseudotuberculosis* happened 1,500-20,000 years ago (126,136), while other sources suggest that the divergence happened 3,000-6,000 years ago in central Asia (136,137). *Yersinia pseudotuberculosis* is is associated with many animal species and environmental sources and is transmitted via the faecal-oral route to cause relatively mild and self-limiting enteric infections. In contrast, *Y. pestis* has evolved the ability to colonize an arthropod vector, the flea, and infects mammalian hosts by subcutaneous and pneumonic routes (126).

Yersinia pestis has been grouped into three main subtypes, or biovars (i.e., antiqua, medievalis, and orientalis), that are biochemically distinguishable from each other in terms of their ability to ferment glycerol and reduce nitrate, by ribotyping, or by pulsed-field gel electrophoresis (PFGE) (138–140). The biovar orientalis has spread worldwide, while the other two are more localized to enzootic rodent hosts in Africa and central Asia (136). Plague primarily affects rodents but humans can be infected through fleabites, contact with rodents, or person-to-person by the respiratory route in the case of pneumonic plague. *Yersinia pestis* invades the subcutaneous site of the fleabite before progressing to the lymph nodes where it multiplies to cause extensive swelling and other symptoms of bubonic plague. Bacteria are then transferred to the liver and the spleen through the bloodstream before finally reaching the lungs, causing pneumonic plague, which is the most severe and contagious form of the disease (141).

2.10.1.2. Major virulence factors of Y. pestis

Yersinia pestis depends on its flea vector and the cyclical transmission pathway between fleas and mammals. It was previously thought that the highly virulent *Y. pestis* emerged from a form of *Y. pseudotuberculosis* that developed the ability to survive inside the flea vector (136). At a point in its evolution, *Y. pestis* acquired a plasmid called pMT1 that includes the *ymt* gene, which encodes a murine toxin. It is highly toxic for mice and rats but less potent in other mammals. The toxin is necessary for *Y. pestis* during colonization of the flea intestine (142). Based on studies of bacterial genomes sequenced from tooth samples of Bronze Age humans, Rasmussen et al. have suggested that fully virulent plague strains originated around 5,000 years ago from *Y. pestis* strains lacking the *Yersinia* murine toxin. They argued that these ancestral strains were able to cause pneumonic and septicemic plague but not the bubonic form (143).

Another plasmid, pPCP1, which is also called the pPla, is present in *Y. pestis* but not in *Y. Pseudotuberculosis*, and plays a role in virulence in mammals (144). Specifically, it encodes a plasminogen activator necessary for the invasion of the host lymph nodes (145). Plasminogen activator is a laminin-specific adhesion protein and works by degrading the main circulating inhibitor of plasmin (the α2-antiplasmin) which leads to proteolysis and thereby enables the spread of *Y. pestis* from the subcutaneous infection site into the bloodstream (141,146,147) *Y. pestis* has also lost a number of genes due to mutation and deletion (148), some genes may still be present in the genome as pseudogenes. Some of the gene losses have been positively selected and enabled the transmission of *Y. pestis* by fleas into the mammalian hosts (137).

2.10.1.3. Y. pestis *as BW agent*

Ingelsby et al. have concluded that plague outbreaks following the use of a biological weapon would be a plausible threat (127). Rapid progression of the disease, lethality, and its ability to be transmitted via aerosol make *Y. pestis* a potential BW/BT agent. The Japanese army Unit 731 is reported to have released plague-infected fleas against China in WWII, causing

outbreaks of plague. Ingelsby et al. also stated that the BW programs of the US and the former Soviet Union developed techniques to aerosolize plague directly, without having to use the flea vector for its dissemination. If the aerosolized form of the *Y. pestis* bacteria would be inhaled due to a biological attack, it would cause primary pneumonic plague and the pathogenesis and clinical manifestations would be different than in natural instances of plague (128). The quantity and characteristics of the strain, environmental conditions during release and the method of aerosolisation would define the size of the outbreak of the disease and the number of victims. In 1970, WHO estimated that if 50 kg of *Y. pestis* was released over a city of 5 million, the bacteria would remain viable in their aerosol form for up to an hour and travel a distance of up to 10 km. As a result, 150,000 people would likely develop pneumonic plague and 36,000 of them would likely die (5). Symptoms with fever, cough and chest pain would begin in 1 to 6 days after exposure followed by rapid progression of the disease, which would lead to septic shock with high mortality rate if the treatment was not started promptly. Appearance of the disease in areas where the disease is not endemic and in persons without known risk factors could indicate a deliberate release of *Y. pestis* (127).

2.10.1.4. Identification, treatment and prophylaxis

Bubonic plague can be diagnosed on the basis of a Gram-negative bacilli lymph node aspirate or biopsy samples. Pneumonic and septicemic plague are more difficult to diagnose, especially in non-endemic areas. In the laboratory, *Y. pestis* can be grown in blood culture and identified with biochemical tests or by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (149). Samples suspected of containing *Y. pestis* should always be handled in a BSL-3 laboratory. When real-time PCR is used, the DNA can be first isolated in BSL-3 and then subsequently analysed in a standard molecular biology laboratory. PCR testing is a suitable diagnostic method when pneumonic plague is suspected (150). In Madagascar and the Democratic Republic of Congo, rapid immunological LF tests have been used to detect *Y. pestis* in sputum samples (151).

Y. pestis is sensitive to a number of antibiotics and can be successfully treated with intravenous or oral ciprofloxacin and doxycycline, but the treatment must be started early since the progression of pneumonic plague is rapid and effective treatment becomes increasingly difficult (127,152). Pneumonic plague progresses into an irreversible and lethal syndrome within 24–36 h after the onset of symptoms and cannot be effectively treated after the lethal symptoms have begun. The mortality rate for this disease approaches 100% (152). Patients with pneumonic plague should be isolated at least for 48 h to prevent infection of medical personnel. In the case of an intentional release, the local flea and rodent populations should also be controlled to help prevent the spread of the disease (153).

In a bioterrorism event or a pandemic scenario, it prophylactic treatment should be given to those at high risk. Currently there is no licensed vaccine against plague, but a new candidate for an oral vaccine based on attenuated *Y. pseudotuberculosis* strain VTnF1 live vaccine that expresses *Y. pestis* F1 antigen has recently been developed (125).

2.10.2 Bacillus anthracis

Bacillus anthracis, the etiologic agent of anthrax, is a Gram-positive spore-forming non-motile bacterium commonly found in soil. The bacterium causes a zoonotic disease associated with herbivores that ingest infective spores while grazing (154,155). Where it occurs naturally, *B. anthracis* can infect humans via infected livestock and their products. The bacteria enter the body through skin lesions, airways or orally where they cause cutaneous, pulmonary or gastrointestinal anthrax, respectively. The most common naturally-occurring form is cutaneous anthrax with an estimated 2,000 annual cases worldwide (156). Anthrax can also result in a systemic infection and lethal disease in mammals. It is one of the most potent BW agents, because it forms endospores that are extremely resistant to environmental conditions, and can survive for several decades in the environment (155). Due to the extremely resistant nature of spores, *B. anthracis* can be disseminated in an aerosolized form or as a powder, as in the 2001 US letter attack.

2.10.2.1. Virulence factor of B. anthracis

Bacillus anthracis belongs to the "*B. cereus* group", which is also called "group 1 bacilli". This group also includes *B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides, and B. weihenstephanensis* (157–163). *Bacillus* species in this group all have a similar cell structure, physiology, and system of genetic exchange. However, their pathogenicity is different and most of the genes controlling virulence are located in plasmids. Unlike other species of the *B. cereus* group, *B. anthracis* synthesizes anthrax toxins. It has two virulence plasmids: pXO1 containing the structural genes for the anthrax toxin proteins (*pagA*, *lef* and *cya*), and; pXO2 containing the biosynthetic operon *capBCADE* encoding the capsule (154). Since the discovery of plasmids pXO1 and pXO2 in the 1980s, they have been considered as the distinctive genetic feature of *B. anthracis* (164–166). For safety reasons, attenuated strains that have only one of the two virulence plasmids are used in research. "Sterne" strains carry the pXO1 plasmid and "Pasteur" strains only have pXO2 (164,167).

2.10.2.2. B. anthracis *as a BW agent*

B. anthracis has been studied for use as a biological weapon for more than eighty years. During its history, at least seventeen nations are believed to have or have had offensive biological weapons programs, but the total number of countries that may have worked with anthrax at some time is unknown (41,168). One country that acknowledged producing and weaponizing anthrax is Iraq (169). Manufacturing lethal anthrax aerosol requires advanced biotechnology capabilities which many believe to be beyond independent or terrorist groups. However, it is possible that some groups could obtain the materials required for an attack. In a classical example of the clandestine production of anthrax spores, aerosolized spores were accidentally released from a Soviet military microbiology facility in Sverdlovsk in 1979, which resulted in at least 79 anthrax cases and 68 deaths (15). Similar to attacks with other BW agents, the anthrax aerosol could not be seen, had no odour and the aerosol could spread a

distance of many kilometres. A WHO committee estimated that following the release of 50 kg of anthrax from an aircraft over a typical city of half a million people, 250,000 would be exposed. Out of these casualties, 95,000 would likely die if they did not receive prompt medical attention (5, 171).

Inhalation of anthrax particles after an intentional aerosol resealse, particles that are 1 to 5 μm in diameter would adhere to the lung surfaces and subsequently be ingested by macrophages (171). Some of the spores would survive lysis in the macrophage and travel to lymph nodes prior to germinating, which can be delayed by up to 100 days (172–175). Following germination, disease symptoms present rapidly as active bacterial cells begin releasing toxins that cause haemorrhage, oedema, and necrosis (175). Based on primate data, the lethal dose sufficient to kill 50% of those exposed to it (LD 50) is 2,500 to 55,000 inhaled anthrax spores (171).

2.10.2.3 Diagnosis and detection

A sudden and acute onset of a flu-like illness in a large number of people is the first indication of a BW attack with anthrax spores, especially if the disease is not known to occur in the region. Fatality rates could be as high as 80% or more in an anthrax attack and nearly half of all deaths would occur within the first two days (32).

Standard blood culture and biochemical testing are used to diagnose anthrax. Many rapid assays based on LF, ELISA for the protective antigen, and PCR assays for the detection of virulence plasmids pXO1 and pXO2 have also been developed for detection and identification of *B. anthracis* (176–179).

2.10.2.4. Prophylaxis, treatment and decontamination

While anthrax vaccines exist, none are available for general use as a prophylactic. The US has an anthrax vaccine for military personnel made from the cell-free filtrate of a non-encapsulated attenuated strain of *B. anthracis.* The vaccine was licensed in 1970. The UK has an anthrax vaccine for humans that was developed in the 1950s, and its use is limited to occupations and situations where the risk of exposure is high (180). In the former Soviet Union, a live attenuated vaccine was produced and used in humans, but in the Western countries live attenuated vaccines have not been considered safe for use in humans, but are used for livestock vaccinations (37,171,180,181). Next generation recombinant sub-unit vaccines have components that are highly defined and which comply with current regulations. Recombinant live vaccines are also being studied at this time (182,183).

Decontamination in the event of an intentional aerosolisation of anthrax spores is difficult and requires considerable effort. This has been demonstrated by the UK testing of explosives containing anthrax spores during WWII on Gruinard island off the coast of Scotland. The

spores remained viable at least for 36 years. Gruinard was declared safe in 1987 after massive decontamination of the island requiring 2,000 tons of seawater and 280 tons of formaldehyde (184,185). During decontamination, the risk of secondary aerosolisation of spores is high and the waste generated is also problematic to dispose of (186).

2.10.3 Francisella tularensis

Francisella tularensis is an aerobic, non-motile, small Gram-negative coccobacillus that causes tularemia that can be fatal in humans. It was first described in the 1910s and studied extensively by Edward Francis (187). *Francisella tularensis* is one of the most infectious pathogenic bacteria known; the infective inhalation dose can be as low as 10 cells. It is a facultative intracellular bacterium that multiplies within macrophages. It can survive for weeks in water, soil, and in decaying animal carcasses at low temperatures (188). It has been previously divided into two main biovars based on virulence testing, biochemical reactions, and epidemiological features. These are *F. t.* biovar tularensis (type A) and *F. t.* biovar *holarctica* (type B). Currently, there are four subspecies recognized, namely *tularensis, holarctica, mediasiatica,* and *novicida*. Subspecies *tularensis* may be highly virulent in humans and animals and is found mainly in North America, while subsp. *holarctica* is milder and occurs mostly in Europe and Asia. *Francisella tularensis* can infect humans through skin, mucous membranes, gastrointestinal tract, or lungs (189,190). *S*ubspecies *mediasiatica* is virulent and has been isolated from animals in central Asia. Subspecies *novicida* is very rare and its ability to cause disease in humans is unclear (191).

2.10.3.1 Transmission of F. tularensis

Tularemia caused by subsp. *holarctica* occurs widely in the Palearctic and subsp. *tularensis* in North America. Most human cases of tularemia are reported from northern and central Europe. Tularemia also occurs in Finland, Scandinavia, countries of the former Soviet Union, China, and Japan (192). In Sweden, there was a large outbreak of airborne tularemia involving more than 600 people 1966-1967 (193). Most of the patients acquired the infection while doing farm work that created contaminated aerosols. In Finland, ten to several hundred cases are reported each year to the infectious disease register at the National Institute of Health and Welfare (THL) since 1995. A peak was seen in 2000 (926 cases) while only 10 cases were reported in 2014 (194). It is likely that the global incidence of tularemia is underestimated due to misdiagnosis or failed detection (195).

Humans are infected by *F. tularensis* through animal hosts such as voles, mice, water rats, squirrels, rabbits and hares by handling infected animal tissues or faeces in agricultural activities, hunting and trapping (196). *Francisella tularensis* can also be found in contaminated water, soil and plants (188). Furtmermore, ticks, flies, horseflies, and mosquitoes carry the infection and may transmit the bacteria to humans but the infection is not transmitted from person to person (191,195).

2.10.3.2 Francisella tularensis *as a BW agent*

Francisella tularensis has long been considered a potentially dangerous BW agent because of its high infectivity (subsp. *tularensis*), ease of dissemination, and capacity to cause illness and death (195,197). The bacterium was studied by Japanese BW research units (731 Manchuria Unit) that operated before and during WWII (198). Western researchers have also been interested in *F. tularensis* and the US military developed weapons in the 1950s and 1960s that could disseminate *F. tularensis* aerosols (168). During WWII, outbreaks of tularemia affected tens of thousands of Soviet and German soldiers on the eastern front, and it has been suggested by Ken Alibek, a scientist who worked with bioweapons in the former Soviet Union, that these outbreaks resulted from the intentional use of *F. tularensis*. He also claimed that the interest in use of tularemia as a BW agent continued after the war and *F. tularensis* strains engineered to be resistant to antibiotics and vaccines were produced in 1990s in the former Soviet Union (63).

An aerosol release of virulent *F. tularensis* would have severe consequences. Similar to other BW agents, the WHO estimated that aerosol dispersal of 50 kg of virulent *F. tularensis* over a city of five million would result in 250,000 infections and 19,000 deaths (5). It would be difficult to distinguish an attack from a natural outbreak such as influenza or atypical pneumonia. However, sudden onset of large numbers of cases, rapid progression to life-threatening and systemic infections that affect young, healthy adults and children, especially in urban areas, should alert doctors and public health authorities to a possible bioterrorist attack (127,171,199).

2.10.3.3. Diagnosis and treatment

Isolation and identification of *F. tularensis* by culture or standard laboratory testing could take several weeks and the laboratory must be equipped with proper biosafety measures due to the low dose required for infection. Antigen testing of blood samples can help (192), but rapid diagnostic tests for tularemia are not widely available (195). Direct examination of patient samples using fluorescent antibodies or immunohistochemical staining, PCR, PFGE, ELISA, immunoblotting and other antigen detection assays may be used to identify and characterize *F. tularensis* strains (200–207). Tularemia can be treated with antibiotics such as streptomycin, gentamicin, doxycycline, or ciprofloxacin, but if left untreated, the disease may lead to respiratory failure, shock, and eventually death. Doxycycline or ciprofloxacin may also be used prophylactically (195).

2.10.4 Brucella

Brucella are small, non-motile facultative Gram-negative bacteria that belong to the class of α-proteobacteria and are able to grow both extracellularly and intracellularly within macrophages. The genus *Brucella* consists of six species (*B. melitensis, B. abortus, B. suis, B. canis, B. neotomae* and *B. ovis*) although some researchers have argued that these are so

closely related that they should actually be regarded as one species based on DNA-DNA hybridisation studies (208), and molecular genetics (209,210) and that they contain highly similar genomes in terms of sequence identity (211–213). While the taxonomy of the group has been debated, Moreno et al. argued in 2002 that classical host range was a valid criterion on which to recognize the different species and understanding their evolution, and many researchers have embraced this approach (214). The six classical *Brucella* species and their biovars remain valid and the pre-1986 taxonomy of *Brucella* was reinstated (215) and additional species have since been described.

2.10.4.1 Prevalence of Brucella *spp.*

Brucellosis is a zoonosis and, as mentioned above, the species are recognized on the basis of their host species (216) (Table 9). Brucella infection can cause abortion and infertility in the host animals. *Brucella pinnipediae* and *B. ceti* have been discovered quite recently from marine mammals (217–219), the latter being a close relative of *B. abortus* and *B. melitensis* (220). *Brucella microti* has been suggested as a new species that can also be isolated directly from soil (216,221,222). The most recent addition to the genus is *B. inopinata* that was isolated from a breast implant wound of a 71-year old patient with symptoms of brucellosis. It was described as a new species by Scholz et al. in 2010 (223).

Table 9. Brucella species and their host range.

Species	Host species	Infects humans
B. melitensis	sheep, goat	yes
B. ovis	sheep, goat	no
B. abortus	cattle	yes
B. suis	pigs	yes
B. canis	dogs, canines	yes
B. neotomae	rodents	no
B. pinnipediae	dolphins, seals	no
B. ceti	striped dolphins	no
B. microti	rodents, foxes	no
B. inopinata sp. nov.	humans	yes

Brucellosis is common worldwide and induces a chronic disease with low mortality, but which is often incapacitating in humans. Human brucellosis is mainly caused by *B. melitensis* in the Mediterranean, Middle East, Central Asia, Africa and Latin America and has been re-emerging during recent years (224). *Brucella* species are highly infective when aerosolised, and the infectious dose is somewhere between 10 to 100 cells, which makes *Brucella* an attractive BW agent (225). It can survive for long periods in food and human infection is usually obtained from unpasteurized dairy products such as milk or soft cheese. However, *Brucella* is sensitive to heat, ionizing radiation, commonly used disinfectants and pasteurization (226). *Brucella* can easily be aerosolized, and brucellosis is also known as the

most common laboratory-acquired infection worldwide (227). Brucellosis is not transmitted from person to person.

2.10.4.2. **Brucella** *as a BW agent*

Brucella spp. are on the category B list of the CDC and the US National Institute of Allergy and Infectious Diseases (NIAID), and included in the lists of potential BW agents produced by WHO, BTWC and NATO (228). It was studied by many nations in offensive BW programmes (Table 10). *Brucella suis* was the first agent weaponised in the US during the 1950s and field-tested with bombs filled with the bacteria (168,228). It was also tested by the Japanese 731 Manchuria Unit during WWII and developed for offensive purposes by the Soviet Union's BW program Biopreparat. Degonay et al. argued that interest in *Brucella* as a biological weapon these days only concerns its historical significance, because it has low mortality and the inoculation period is long, i.e., from 9 days to 6 months (225,228).

2.10.4.3. Symptoms and diagnosis

Brucellosis resulting from a bioterrorism event would be characterized by a gradually increasing number of cases similar to a natural outbreak. Symptoms may be acute or chronic, intermittent or irregular fever and profuse sweating, fatigue, anorexia, weight loss, headache and generalized aching. Unexpected and clustered cases of brucellosis in the human and animal population should alert local health authorities to an intentional release (225).

Isolating the organism by culture is the gold standard for *Brucella* diagnosis, but the process from unknown sample to confirmed identification may take from three days to several weeks. Blood and bone marrow culture have been considered to be extremely sensitive diagnostic methods (229,230). Serological testing (e.g., surface antigen agglutination tests) have been a widely applied screening method for the diagnosis of *Brucella* infection, but these tests may suffer from false-negative or false-positive results due to cross-reactions. ELISA tests are more sensitive than serum agglutination tests. Culturing and testing the agent is hazardous to laboratory workers due to the low infectious dose and ease of aerosolisation. RtPCR is fast and less hazardous for laboratory personnel than culturing and has become an important tool in *Brucella* diagnostics (231–235). In some cases, e.g. diagnosis of human cases or food contamination, genus-specific PCR assay is sufficient, but in other instances, such as epidemiological surveys, a species-specific identification is needed (231).

Several genus-specific PCR or rtPCR assays that detect the 16S rRNA gene (236–238), 31 kD *Brucella* cell surface protein, *bcsp31* gene (239–241), and *per* gene of perosamine (242) have been developed. The insertion sequence IS711 has also been shown to be a highly sensitive and specific target for the genus *Brucella* (243). Grote et al. have patented a specific test kit for detecting *B. melitensis* and *B. abortus* from environmental samples (244).

2.10.4.4. Vaccines and treatment

Unfortunately, no safe and effective human brucellosis vaccine currently exists, although there are many on-going studies and many vaccines have been tested, especially in the former Soviet Union (225). However, live attenuated strains of *B. abortus, B. melitensis* and *B. suis* have been used effectively in the vaccination of livestock. Vaccination would be very important in post-exposure treatment and for those deemed to be at risk of infection (225,227,245). Doxycycline and rifampin are recommended antibiotics to be administered for at least six weeks. However, therapeutic failure and relapse are possible (226,227,246). In severe cases, such as meningitis or endocarditis, a variety of treatments and medicines may need to be used (228).

2.10.5. Vibrio cholerae

Cholera is an acute intestinal infection caused by a waterborne Gram-negative motile bacterium *Vibrio cholerae*. It occurs in more than 50 countries worldwide and causes large epidemics, especially in equatorial Africa, Asia and the Caribbean. The disease has a rapid onset and it causes a severe watery diarrhoea, vomiting and dehydration which can lead to kidney failure and sudden death if left untreated. It has a very short incubation, from two hours to five days, and can kill even healthy adults within hours (247). The two major serogroups of *V. cholera* that cause epidemics are O1 and O139, while others (non-O1 and non-O139) have been associated with occasional outbreaks (107,248).

The original reservoir of *V. cholera* is in the delta region of the Ganges River in India, but it now occurs in aquatic ecosystems worldwide. Brackish (i.e., estuarine) water habitats and humans are the main reservoirs of the bacteria. It is often contracted from contaminated water or seafood such as mussels or shrimps. Huq et al. have shown that zooplankton acts as a host to commensal bacterium and enables the persistence and multiplication of pathogenic O1 *V. cholerae* in aquatic environments. As a consequence of this, cholera outbreaks in Bangladesh are associated with the seasonal zooplankton bloom in September and October (249,250). The disease spreads rapidly through the faecal–oral route and fresh stool is especially infective (251–254). Cholera has caused six pandemics and killed millions of people in Europe, and the seventh pandemic that began in 1961 in South-east Asia is still on-going. Recent studies suggest that climate change might create a more favourable environment for *V. cholera* in areas where sanitation is poor, since cholera is mainly transmitted through contaminated food and water. Beginning in 2005, cholera has re-emerged in response to as an increasing number of people living in urban slums, disaster zones or refugee camps as a consequence of man-made or natural catastrophies. Temporary camps have poor infrastructure and limited or no access to clean water (247). In 2011, a total of 589,854 cholera cases were reported to WHO from 58 countries. These led to 7,816 deaths and the case fatality rate (CFR) was therefore 1.3%. Most cholera cases are reported from Africa, but in 2011, 61% of the cases were from a large outbreak that affected Haiti and the Dominican Republic following the 2010 earthquake. The number of actual cases worldwide is much

higher than reported due to limitations on surveillance and reporting systems, and inconsistencies in case definitions and terminology (77).

2.10.5.1. Subtypes and virulence factors of V. cholerae

The serogroup O1 is divided into three serotypes (Inaba, Ogawa and Hikojima) and these are further divided into two biotypes (classical and El Tor) (248,255,256). The severity of clinical symptoms and the expression and regulation of major virulence factors of each biotype are different (256). The current pandemic is caused by the El Tor biotype of the O1 serogroup, with periodic emergence of serogroup O139 that is prevalent in Asia (254,257). *Vibrio cholerae* can alternate between motile and biofilm lifestyle, which helps it to efficiently colonize the small intestine. After entering the host body, *V. cholera* moves by chemotaxis to the small intestine, where it attaches. During infection it can form biofilm-like structures that are more resistant to the hostile conditions inside the host. The formation of a biofilm and dispersal during infection may enhance dissemination of *V. cholerae* along the small intestine and its transmission to a secondary host through the faecal–oral route (254). The major virulence factors of *V. cholera* are cholera toxin (CT) and the toxin-coregulated pilus (TCP) that are encoded by ctxAB and tcpA genes (256). Faruque and Nair conclude in their review that acquisition of virulence genes by the marine or brackish water bacterium was a prerequisite for adaptation to the human intestine. This could, in part, explain the increased evolutionary fitness of pathogenic strains of *V. cholerae* (258).

2.10.5.2. Diagnosis of cholera

The conventional diagnostic methods for cholera are based on culture, microscopy and biochemical testing. However, *V. cholera* can enter a viable but nonculturable (VBNC) dormant state, which is a survival strategy in the natural aquatic environment of the microbe (256). As for other pathogens, PCR and other molecular methods have become more important in the detection and diagnosis of cholera. This has improved and accelerated the decision-making process in relation to infection control, and treatment (62).

Several monoplex and multiplex PCR tests have been developed for toxigenic, serogroup O1 and O139 *V. cholera* detection since the 1990s (124,212,259–262). Similarly, tests for environmental non-O1/non-O139 serogroups and tests that detect all serogroups of *V. cholerae* have been developed (263,264). The non-O1/non-O139 serogoups do not possess the toxin or other virulence genes but are responsible for some sporadic and localized outbreaks of cholera-like diarrhoea. Chua et al. have developed a cold chain-free, lyophilized, ready-to-use triplex PCR kit for detecting *V. cholera* that they claim is ready and easy to deploy in the event of a suspected cholera outbreak (265).

2.10.5.3. Cholera prophylaxis and treatment

The toxigenic *V. cholerae* is listed by the CDC as a category B biothreat agent because it could be used to deliberately contaminate water or food and cause intentional harm (257), major disruption to society, or incapacitate a confined army. Treatment of cholera is prompt use of oral rehydration fluids and salts or, in severe cases, intravenous fluids. Oral rehydration salts are sufficient for up to 80% of patients. Erythromycin, tetracyclin or doxycycline antibiotics may then be used to kill the pathogen and diminish the duration of diarrhoea. This reduces the need for more rehydration fluids, and shortens the duration of excretion of the organism in faeces. WHO recommends antibiotic treatment only in severe cases (266). Three oral cholera vaccines have been approved, but only one of them has an international licence (Ducoral®, Crucell, the Netherlands) (267).

2.11 *Clostridium difficile*

Clostridium difficile is a Gram-positive anaerobic bacillus that forms spores which are highly resistant to physical (i.e., heat, dessication) and chemical disinfectants. It is not on any of the BT/BW lists currently produced, even thought it is closely related to *Clostridium botulinum* that produces the one most potent toxins known — the botulinum toxin. However, *C. difficile* is an important pathogen that has emerged in the antibiotic era and for which the development of faster, specific and more sensitive diagnostics has been an issue since the increase of its incidence from the early 2000s. In this study, *C. difficile* was used as an agent for which a newly-developed small and portable diagnostic device was tested in an accuracy study with clinical patient samples in two European countries and three hospital laboratories.

Clostridium difficile can be found in soil and in the gastrointestinal tract of many animals such as pigs, cows, horses and dogs, and humans, as well as in food products (268,269). It was first discovered in 1935 by Hall and O'Toole as a part of the normal flora of new-born infants (270). *Clostridium difficile* was not associated with a human disease at that time, and it was not considered to be important as a pathogen until the antibiotic era (271). In 1978 Tedesco et al. and Bartlett et al. identified *C. difficile* as the causative agent of pseudomembranous colitis in patients receiving clindamycin (272–274).

These days *C. difficile* is the leading cause of healthcare-associated diarrhoeal infections and colitis in the industrialized world (274). It is transmitted via the faecal-oral pathway. It has been shown that the healthcare-associated *C. difficile* infections (CDIs) are transmitted mostly by spores (275). The spores produced by *C. difficile* are highly resistant to disinfectants and can exist on surfaces in hospitals for long periods of time. The infection is spread via contact with contaminated surfaces, through the hands of health care workers, or by direct patient-to-patient contact. Routine hand-washing and thorough cleaning of facilities, personnel and equipment is of paramount importance for preventing the spread of the disease. It should be noted that antiseptic hand cleaning products based on alcohol are inefficient at killing the *C. difficile* spores (276,277). CDI can lead to diarrhoea, pseudomembranous colitis, toxic

megacolon and even death, but *C. difficile* can also colonize the human gut as an asymptomatic inhabitant. The asymptomatic colonization rate is estimated to be as high as 60-70% in infants and 3-15% in healthy adults (278,279). According to some sources, the majority of newborns are colonized with *C. difficile* in the first weeks of life and the colonisation rate decreases to around 10% by one year of age, and by age three the percentage is similar to that of asymptomatic adults (280). It has been suggested that newborns and infants may lack the *C. difficile* toxin receptors, and therefore not develop CDI even when colonized (281–284). Colonisation rates in nursing homes and among hospitalized patients is estimated to be 25-55%, and newly-admitted patients carrying *C. difficile* may serve as a source of re-infection (285,286).

2.11.1. Risk factors of CDI

The risk factors for acquiring CDI are usage of broad-spectrum antibiotics, especially a course of fluoroquinolones, clindamycin, cephalosporines or penicillins, and hospitalization, advanced age and other diseases (287–289). Using products that decrease the acidity of the stomach, such as proton pump inhibitors and H2 blockers, may also promote the development of CDI by allowing *C. difficile* spores to pass through the stomach to the intestines, where the anaerobic environment and presence of bile salts allow germination of the spores into the vegetative form and the production of toxin, leading to diarrhoea (290–293). Approximately 10% of patients receiving antibiotics experience antibiotic-associated diarrhoea; of these patients CDI accounts for about 20% (294).

Incidence of CDI has increased dramatically since 2000, which has been attributed partly to the emergence of new strain types especially the so-called hypervirulent BI/NAP1/027 strain (271,295). This ribotype ("027") belongs to toxinotype III and has a higher level of toxin production (296,297) and resistance to fluoroquinolone antibiotics (298). The rate of community-acquired CDI affecting people considered to be at at low risk, younger, unhospitalized and not on antibiotics has also increased (295). According to recent surveillance data, approximately 20% or more of all CDI cases are community-associated (299,300).

2.11.2 Major virulence factors of C. difficile

It has been estimated that the human gut contains 10^{14} bacterial cells, which represent thousands of bacterial species belonging to the phyla of Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, Proteobacteria and Fusobacteria (301). The microbiological diversity in the colon is maintained by these bacteria together with protozoans, fungi and bacteriophages (302). If the balance of the intestinal microbiome is disturbed by antibiotic use, especially administration of a long courses of antibiotics, it can lead to colonization of the gut by the toxigenic *C. difficile* and lead to CDI (303). The main virulence factors of *C. difficile* are two large clostridial endotoxins, toxin A and toxin B (TcdA, 308 kDa; TcdB, 270 kDa),

encoded by the genes *tcdA* and *tcdB* that are part of the *C. difficile* pathogenicity locus (PaLoc). PaLoc also contains the genes *tcdC*, *tcdR* and *tcdE* (304). Different *C. difficile* strains have variable deletions in the PaLoc region, and can be divided into different toxinotypes based on this variation (305). Some strains contain deletions in the toxin genes and fail to produce the toxins thus do not cause infection. A third toxin, know as binary toxin or *C. difficile* transferase (CDT), is produced by some strains, but the significance of this toxin in the disease remains uncertain (271).

2.11.3. Diagnosis of CDI

Professional societies, such as the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), have published guidelines for the diagnosis and treatment of CDI. The recommendation is that any hospitalized patient with ≥3 unformed stools in a twenty-four hour period should be tested for possible *C. difficile* infection. Only watery, liquid, or unformed stools should be tested. According to the recommendations, asymptomatic patients should not be tested, not even to test that they have been cured, since they may still shed *C. difficile* DNA for some time after they no longer have symptoms of the disease (306–309).

Many laboratories have previously used rapid enzyme immunoassays (EIA) for the detection of glutamate dehydrogenase (GDH) and/or *C. difficile* toxin or toxins for the diagnosis of *C. difficile*, although it is now generally accepted that the rapid assays available are not very sensitive (271). Although variable performance of GDH tests has also been reported, the test appears sensitive to *C. difficile* but cannot discriminate between toxigenic and non-toxigenic strains (310). Other commonly used tests are toxigenic culture (TC), which consists of *C. difficile* from a faecal sample grown on selective media followed by toxin detection, cell culture cytotoxicity assay (CCTA) and molecular amplification assays. There are several molecular assays commercially available for the detection of toxigenic *C. difficile.* Most of the molecular tests target the toxin B gene (*tcdB*) (311). Peterson et al. suggested that rtPCR for *tcdB* detection is the most sensitive and specific test for detecting the presence of toxigenic *C. difficile* in the stool of undiagnosed patients. The other advantage is that results from molecular tests can be available the same day the sample is collected, while the toxigenic culture and cell cytotoxicity assay may take several days to complete (312). However, the clinical picture must always be taken into account as the detection of *C. difficile* in a faecal specimen does not always imply disease and may also be due to asymptomatic carriage and colonization with a toxigenic strain (312,313). In some situations (e.g., diagnosis of *C. difficile* in nursing homes), easy-to-use POC tests could help detect a local outbreak and trigger isolation and decontamination protocols. While not being a BT/BW agent, *C. difficile* is an important pathogen and development of easy-to-use tests for its diagnostics is important for health care professionals.

2.11.4. Treatment of CDI

A range of different treatments is currently known and being pursued for handling CDI. The antibiotics metronidazole, vancomycin and a newer antibiotic fidaxomicin, are the first line of treatment for CDI, after the cessation of the antibiotic treatment that is the cause of the disturbance of the normal microbial flora of the intestine, in the case that this is medically appropriate. (314–316). New therapies, such as faecal transplants and probiotics, which are aimed at restoring the normal microbiome in the intestine, are becoming increasingly used. Vaccines for CDI are currently being tested in clinical trials (308,317,318). Faecal microbiota transplantation (FMT) has actually been used to treat patients with pseudomembranous colitis already for several decades, the first case was reported in 1958 (308,319). In a recent study, Mattila et al. have shown that FMT is an effective treatment for recurrent CDI (320). Mattila et al. also performed a preliminary study comparing *C. difficile* immune whey (CDIW) versus metronidazole treatment in patients with recurrent episodes of CDI. The immune whey was produced from the colostrum of cows immunized with *C. difficil*e vaccine during their gestation period, and subsequently collecting and concentrating the immunoglobulins into the final whey product. Although the group had to interrupt the trial early due to a funding issue, they could present data supporting the hypothesis that CDIW was as effective as metronidazole for the prevention of recurrent CDI, and was well tolerated by the patients (321). The treatment for recurrent CDI with FMT has not become a routine procedure so far, most likely due to the rather unappealing nature of the treatment, and a limited number of data from randomized controlled trials to prove the efficacy and safety of it (308).

3. AIMS OF THE STUDY

Pathogenic bacteria can cause severe disease in humans, whether they are naturally occurring or intentionally spread in an act of terrorism or warfare. The purpose of this study was to assess and develop methods and platforms for rapid and accurate detection of potentially harmful pathogenic bacteria from environmental or patient samples utilising molecular methods. The aim was to determine the best field-deployable methods and instruments that would be fast and easy to use.

The specific aims of this study were:

1. To develop an accurate multiplexed molecular detection method for *Y. pestis* and *Y. pseudotuberculosis*

2. To develop a multiplatform molecular based assay for the detection of *V. cholera* from field samples

3. To transfer molecular assays for *B. anthracis*, *F. tularensis* and *Y. pestis* to a field-deployable instrument and to compare its performance to a laboratory-based instrument utilising these assays. Furthermore, the aim was to develop an assay for *Brucella* spp., and a simple assay for field use with which to train personnel in the detection and diagnosis of biological agents from powder samples

4. To evaluate an isothermal amplification technology for use in a portable instrument using patient samples for the detection and identification of toxigenic *Clostridium difficile* in a multinational accuracy study

4. MATERIALS AND METHODS

4.1 Bacterial cultures

The bacterial strains or purified DNA of the strains used in study I and in study II and III are given in tables 10 and 11, respectively. Depending on species, bacteria were grown in BSL-2 or BSL-3 laboratories using approved procedures and PPE. In the BSL-3, this included a protective single-use coverall over washable laboratory underclothes, double gloves, goggles, FFP3 respiratory protection and laboratory shoes. The DNA was isolated as described below or in some cases the strains were received as purified DNA for assay verification from the source laboratories, as indicated in the tables below.

4.2 DNA isolation

In study I, bacterial DNA purified previously by the cetyltrimethylammonium bromide (CTAB) method (322) from the EV76 vaccine strain of *Y. pestis* and the various *Y. pseudotuberculosis* strains was used. DNA samples were stored at −20 °C at a final concentration of 50 ng/ml in ddH_2O. Bacterial DNA originating from the clinical sample collection of HUSLAB in study I, as well as the *Vibrio* strains used in study II, were purified with the MagNAPure LC System (Roche, Basel, Switzerland) using the MagNAPure LC DNA isolation kit III according to the manufacturer's instructions. The concentration and purity of the isolated DNA was measured using an Eppendorf DNA calculator or Biophotometer (Eppendorf, Hamburg, Germany). In study I, DNA from 80 consecutive clinical stool samples were collected in September 2007 as well as from the normal microbial flora of the intestine was purified using the EasyMAG system (bioMérieux, Marcy l'Etoile, France) and used for validation experiments of the *Y. pestis*/*Y. pseudotuberculosis* multiplex real-time PCR.

DNA from strains of *B. anthracis, F. tularensis, Y. pestis* and *B. thuringiensis* used in study III and the non-vibrio strains used in study II were purified using the automated MagNA Pure Compact instrument (Roche, Basel, Switzerland) and the MagNA Pure Nucleic Acid Isolation Kit I following a proteinase K digestion in MagNA Pure Bacterial Lysis Buffer according to the manufacturer's instructions. DNA from the clinical *Brucella* isolates was isolated with the Qia-Amp DNA miniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the isolated DNA was measured with the Nano-Drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Table 10. Bacterial strains used in study I and their growth conditions. All *Yersinia* strains were from the laboratory collection of the Department of Bacteriology and Immunology, University of Helsinki.

Bacterial strain	Description	Growth conditions
Y. pestis		
EV76	Vaccine strain	LB, RT, 2-4d
KIM D27	Attenuated strain	LB, RT, 2-4d
Y. pseudotuberculosis		
St. 1	Serotype O:1a	LB, RT, 2-4d
Pa3606	Serotype O:1b	LB, RT, 2-4d
Kuratani-2	Serotype O:1c	LB, RT, 2-4d
208	Serotype O:2a	LB, RT, 2-4d
1779	Serotype O:2b	LB, RT, 2-4d
274	Serotype O:2c	LB, RT, 2-4d
83	Serotype O:3	LB, RT, 2-4d
51	Serotype O:4a	LB, RT, 2-4d
Pa3422	Serotype O:4b	LB, RT, 2-4d
204	Serotype O:5a	LB, RT, 2-4d
197	Serotype O:5b	LB, RT, 2-4d
DD110	Serotype O:6	LB, RT, 2-4d
257	Serotype O:7	LB, RT, 2-4d
151	Serotype O:8	LB, RT, 2-4d
R708	Serotype O:9	LB, RT, 2-4d
6088	Serotype O:10	LB, RT, 2-4d
R80	Serotype O:11	LB, RT, 2-4d
MW864-2	Serotype O:12	LB, RT, 2-4d
N916	Serotype O:13	LB, RT, 2-4d
CN3	Serotype O:14	LB, RT, 2-4d
93422	Serotype O:15	LB, RT, 2-4d
Y. bercovieri 3016/84	Serotype O:58	CLED, RT, 2d
Y. ruckeri RS41		LB, RT, 2d
Y. mollareti 92/84	Serotype O:59	LB, RT, 2d
Y. enterocolitica 1309/80	Serotype O:6	CLED, RT, 2d
Y. intermedia 9/85	Serotype O:16	CLED, RT, 2d
Y. frederikseni 38/83	Serotype O:48	CLED, RT, 2d
Y. kristenseni 119/84	Serotype O:12	CLED, RT, 2d
Agrobacterium tumefaciens C58C1/RP4[a]		CLED, RT, 2d
Pseudomonas aeruginosa IATS[a]	Serotype O:5	CLED, RT, 2d
Enterobacterium cloacae tks461[a]		CLED, +37°C, 2d
Francisella tularensis T-31355[a]		Chocolate agar, 35°C, 5%CO_2, 4d
Streptococcus mitis T-24934 [a]		Blood agar, +35°C, 5%CO_2, 2d
Staphylococcus epidermidis T-15507[a]		Blood agar, +35°C, 5%CO_2, 2d
Micrococcus luteus T-90405[a]		Blood agar, +35°C, 5%CO_2, 2d
Peptostreptococcus acnes [a]		Blood agar, +35°C, 5%CO_2, 2d

LB = Luria-Bertani, CLED = cysteine lactose electrolyte-deficient agar, RT = room temperature, d = days

[a]Source: HUSLAB, the Hospital District of Helsinki and Uusimaa

Table 11. Bacterial strains used in study II and study III, their growth conditions and sources. All of the *V. cholera* strains were cultivated in TS agar.

Bacterial strain, code or serotype	Growth conditions / Comments	Source	Used in
Agrobacterium rhizogenes			
HAMBI 1816 T	DNA	1	III
Agrobacterium tumefaciens			
HAMBI 1811 T	DNA	1	III
Agrobacterium vitis			
HAMBI 1817 T	DNA	1	III
Bacillus anthracis	BHI, +37°C,		
7702	pXO1-/pXO2-	2	II, III
ATCC 4229	pXO1-/pXO2+	2	II, III
Bacillus cereus			
HAMBI 250, ATCC 10987	Nutrient agar, +30°C	1	II
ELMI 21	R1 agar, +30°C	2	III
Bacillus licheniformis			
ELMI 325	R1 agar, +30°C	2	III
Bacillus mycoides			
ELMI 44	R1 agar, +30°C	2	III
Bacillus thuringiensis	TSA		
ssp. *kurstaki-aizaway*	Commercial product	3	III
ELMI 123	R1 agar, +30°C, 2 d	2	III
Bradyrhizobium japonicum			
HAMBI 2314 T	DNA	1	III
Brucella spp.	8 clinical isolates	4	III
Brucella abortus			III
7	biotype 6, DNA	5	III
91	biotype1, DNA	5	III
101	biotype 3, DNA	5	III
120	biotype 4, DNA	5	III
86/8/59/ weybridge	biotype 2, DNA	5	III
NTCC 10093	biotype 1, DNA	5	III
NTCC 10501	biotype 2, DNA	5	III
NTCC 10502	biotype 3, DNA	5	III
NTCC 10503	biotype 4, DNA	5	III
NTCC 10504	biotype 5, DNA	5	III
NTCC 10505	biotype 6, DNA	5	III
NTCC 10506	biotype 7, DNA	5	III
NTCC 10507	biotype 9, DNA	5	III
Brucella melitensis			III
1	biotype 2, DNA	5	III
11	biotype 1, DNA	5	III
72	biotype 3, DNA	5	III
NCTC 10094	biotype 1, DNA	5	III
NCTC 10508	biotype 2, DNA	5	III
NCTC 10509	biotype 3, DNA	5	III
Brucella melitensis			
681	clinical isolate, DNA	4	II
Brucella suis			III
9	biotype 2, DNA	5	III
9/2002	biotype 1, DNA	5	III

NCTC 10510	biotype 2, DNA	5	III
NCTC 10511	biotype 3, DNA	5	III
Brucella canis			III
125	biotype 1, DNA	5	III
RM 6/66 ATCC 23365	biotype 1, DNA	5	III
Brucella neotomae			III
NTCT 10084 SK33	DNA	5	
Brucella maris		5	III
2410	DNA		
2411	DNA		
NTCT 12890	DNA		
NCTC 12891	DNA		
Burkholderia multivorans			
DSM 13243	DNA	6	II
Burkholderia pseudomallei			
HAMBI 33	DNA	1	III
Campylobacter jejuni			
E1 2702/1/04	TS agar, 5% CO_2, +37°C	2	II
Campylobacter upsaliensis			
GNS2897	TS agar, 5% CO_2, +37°C	2	II
Enterobacter cloacae			
tks461	DNA	7	III
Escherichia coli	LB agar, +37°C		
RH 4266	ETEC	8	II
Francisella philomiragia			
DSM 7535	DNA	6	III
Francisella tularensis			
LVS (ATCC 29684)	McLeod agar, 37°C, 5% CO_2	9	II
Listonella anguillarum			
2271/1	LB agar, +25°C	2	II
Mesorhizobium huakuii			
HAMBI 2035 T	DNA	1	III
Mesorhizobium loti			
HAMBI 1129 T	DNA	1	III
Microbacterium barkeri			
DSM 20145	DNA	6	III
Moraxella cararrhalis			
035E	DNA	7	III
Staphylococcus aureus			
ATCC 25923	DNA	7	III
Ochobacterium anthropic			
HAMBI 2402	DNA	1	III
Rhizobium calligum			
HAMBI 2326 T	DNA	1	III
Rhizobium galagae			
HAMBI 540 T	DNA	1	III
Rhizobium legiminosarum			
HAMBI 14 T	DNA	1	III
Salmonella typhimurium			
ATCC 13311	DNA	8	II
Serratia marcescens	TS agar, +30°C		

DSM 1608		6	II
DSM 30121 (ATCC 13880)		6	II
DSM 30126		6	II
DSM 46342 (ATCC 27117)		6	II
Sinorhizobium meliloti			
HAMBI 2148 T	DNA	1	III
Vibrio cholerae	TS agar, 2.5% NaCl, +30-37°C		
VC 2		10	II
VC 4		10	II
VC 5		10	II
VC 6		10	II
VC 14		10	II
VC 18		10	II
VC 21		10	II
VC 24		10	II
VC 27		10	II
VC 37		10	II
VC 45		10	II
VC 46		10	II
VC 47		10	II
VC 48		10	II
VC 61		10	II
VC 69		10	II
VC 84		10	II
VC 86		10	II
VC 110		10	II
VC 117		10	II
VC 134		10	II
VC 161		10	II
VC 177		10	II
VC 198		10	II
VC 211		10	II
VC 216		10	II
VC 229		10	II
VC 230		10	II
VC 232		10	II
VC 233		10	II
VC 234		10	II
VC 235		10	II
VC 236		10	II
VC 237		10	II
VC 238		10	II
VC 239		10	II
VC 240		10	II
VC 241		10	II
VC 242	O1, Ogawa El Tor	10	II
VC 243	O1, Ogawa El Tor	10	II
VC 246		10	II
VC 250		10	II
VC 285		10	II
VC 286		10	II

VC 293		10	II
VC 328		10	II
VC 329		10	II
VC 330		10	II
VC 344		10	II
VC 347		10	II
VC 348		10	II
VC 354		10	II
VC 358		10	II
VC 359		10	II
VC 503		10	II
VC 504		10	II
VC 518		10	II
VC 552		10	II
VC 553		10	II
VC 570		10	II
VC 571		10	II
VC 572		10	II
VC 614		10	II
Vibrio alginolyticus		10	
VA 054	blood agar, +30-37°C	10	II
VA 647	blood agar, +30-37°C	10	II
Vibrio fisheri			
HAMBI 2941	DNA	1	II
Vibrio fluvialis			
VF 062	blood agar, +30-37°C	10	II
Vibrio mimicus		10	
VM 052	blood agar, +30-37°C	10	II
VM 116	blood agar, +30-37°C	10	II
Vibrio metchnicovii			
VM 116	blood agar, +30-37°C	10	II
Vibrio parahemolyticus			
VP 160	blood agar, +30-37°C	10	II
Yersinia bercovieri			
3016/84	See table 10.	7	III
Yersinia enterocolitica			
1309/80	See table 10.	7	III
20373/79		7	II
Yersinia frederikseni			
38/83	See table 10.	7	III
Yersinia intermedia			
9/85	See table 10.	7	III
Yersinia kristensenii			
119/84	See table 10.	7	III
Yersinia mollaretii			
92/84	See table 10.	7	III
Yersinia pestis			
EV76	See table 10.	7	II, III
KIM D1		7	III

Yersinia pseudotuberculosis			
H305-36/89	See table 10.	7	III
No.90		7	III
Yersinia ruckeri			
RS41	See table 10.	7	III

Strain sources: 1. Department of Applied Chemistry and Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Finland, received as purified DNA; 2. Laboratory Strain Collection, Finnish Food Safety Authority Evira; Helsinki, Finland 3. Purified from a commercial insecticide; 4. Turku University Central Hospital and Helsinki District Central Hospital Laboratory Diagnostics HUSLAB; Turku & Helsinki, Finland 5. Institute of Microbiology, German Armed Forces, Munich, Germany, received as purified DNA; 6. DSMZ, German Collection of Microorganism and Cell Cultures, Braunschweig, Germany; 7. Laboratory Strain Collection, Department of Bacteriology and Immunology, University of Helsinki; Finland 8. National Institute for Health and Welfare, Helsinki, Finland 9. Laboratory Strain Collection, FOI, Umea, Sweden; 10. Norwegian Veterinary School of Science, Oslo, Norway. ETEC = enterotoxigenic *E. coli*, LB = Luria-Bertani, TS = tryptone soy agar; BHI = Brain Heart Infusion

4.3 Target selection for the real-time PCR assays

4.3.1 Multiplexed assay for Y. pestis *and* Y. pseudotuberculosis *(Study I)*

For the specific detection of *Y. pestis*, two genomic targets were selected: *pla* and *ypo2088* genes. The *pla* gene encodes for plasminogen activator, which is necessary for the virulence of *Y. pestis* infecting via the subcutaneous route. The *pla* gene is situated in the 9.5 kb pPla-plasmid, and each bacterial cell may contain 150-200 copies of this plasmid (323). Therefore, an assay that detects the *pla* gene has multiple targets per cell and can thus be very sensitive for detecting even small amounts of *Y. pestis* cells present in a sample. The *pla*-specific PCRs have been previously shown to be highly specific (150,324–328). However, since plasmids can be lost during culture when there is no selective force operating in their favour, and some naturally-occurring atypical strains have been isolated from voles in Caucasia that do not harbour the pPla plasmid (325,329), we also developed a protocol for a gene located in the chromosome of *Y. pestis*. This target was the *ypo2088* gene that encodes for a putative methyltransferase and was shown by Zhou et al. to be a specific marker for *Y. pestis* (330).

For the simultaneous detection of *Y. pestis* and *Y. pseudotuberculosis* with one primer and probe set, we decided to use a gene from the lipopolysaccharide (LPS) O-antigen cluster, the *wzz* gene that is common in both bacterial species. In order to do this, we first sequenced the wzz gene fragments of those *Y. pseudotuberculosis* O-serotypes for which the sequence was not known, i.e., serotypes O:6, O:10, O:13, O:14 and O:15. Sequencing of the purified amplicons was performed by the sequencing core facility of the Haartman Institute, Helsinki, Finland. Peter Reeves (Sydney, Australia) kindly provided us the unpublished *wzz* gene sequences for serotypes O:2b, O:2c, O:3, O:4a, O:5a, O:5b and O:7. These sequences were aligned using the ClustalW program with sequences of other serotypes retrieved from the National Center of Biotechnology Information (NCBI) sequence database in order to examine phylogenetic relationships and genetic distances between the O-serotypes of *Y. pseudotuberculosis* and *Y. pestis*.

An internal amplification control (IAC) assay was included in the multiplex in order to monitor reagent performace when other targets were absent. This way, the assay simultaneously detects four targets, namely *pla, ypo2088* and *wzz* genes and the lambda phage IAC. All the primers and probes were designed by TIB Molbiol (TIB Molbiol, Berlin, Germany) based on sequences provided to them. The IAC primers and probe were based on previously published assays (331).

4.3.2 V. cholerae *assays (Study II)*

For the *V. cholerae* assay, two specific targets were selected. The first target was the toxin transcriptional activator gene (*toxR*), which encodes the primary regulator of ctx-tcp operon. The operon itself encodes two of the most important virulence factors of *V. cholera*, the highly potent enterotoxin (CTX) and the toxin-regulated pili (Tcp), which is used by the bacterium as attachment fimbriae. The encoding gene of ToxR regulator, *toxR*, is also present in non-pathogenic strains of *V. cholera*, and was used in this study to detect all *V. cholera* strains including the non-pathogenic environmental strains, while the *ctx* gene was used for distinction of the enterotoxigenic O1 strain of *V. cholerae* that is associated with cholera epidemics and pandemics. The target sequences were determined from conserved homologous regions in sequences available in the NCBI sequence database at the time of designing the assays, and were compared using the Basic Local Alignment Search Tool (BLAST). The primers and probes were designed with the Primer Express software 3.0 (Applied Biosystems, Foster City, CA, USA) and synthetized by Applied Biosystems.

4.3.3 Brucella *spp. and* B. thuringiensis *ssp.* kurstaki *assays (Study III)*

Primers and TaqMan® probes were designed using Primer Express software versions 2.0 and 3.0, and were synthesized by Applied Biosystems. Several primer pairs and probes were initially designed and screened for the genus-specific *Brucella* Assay. Finally, an assay based on *Brucella melitensis* strain 16M insertion sequence IS711 that had the best performance with regards to analytical sensitivity and speed was selected for further studies. This sequence is highly conserved in the genus *Brucella*, but each species has a variable copy number in varying locations of their genomes (332–334). IS711 has been used as a target also in previous *Brucella* species-specific rtPCR assays (240,335).

Bacillus thuringiensis is used in insecticidal products for protection of crops from harmful insects. The pesticidal action is based on the ability to produce crystal toxins, which are encoded by the crystalline genes (*cry* family) (336–338). The c*ry1Ac* gene of *B. thuringiensis* ssp. *kurstaki* was used as a target in a field-training assay developed for spore detection with hoax “anthrax letters” in mind. The primers designed were analysed with the BLAST algorithm via the NCBI GenBank database and predicted to be specific for *B. thuringiensis* ssp. *kurstaki* based on sequences available at the time. Due to a low level of sensitivity and discrepancies across tested platforms, a partial *cry* target from the test sample was sequenced

and one to two nucleotide mismatches were found in the primer and probe sequences. The primers and probes for the *cry* assay were then subsequently re-designed to match the sequencing data.

The primers and probes used in this study are described in tables 12 and 13.

Table 12. Primers used in this study.

Primer	Target organism	Target gene	Sequence (5'-> 3')	Reference
Y. pestis F	*Y. pestis*	*pla*	GCC AAT GAA TAT GAC CTC AAT GTG A	I
Y. pestis S	*Y. pestis*	*pla*	ATG TGA AAG GCT GGT TAC TCC AGA	I
Y. pestis A	*Y. pestis*	*pla*	CCT GCA AGT CCA ATA TAT GGC ATA GA	I
Y. pestis R	*Y. pestis*	*pla*	ATG CGA TAC TGG CCT GCA AG	I
YPO2088 F	*Y. pestis*	*ypo2088*	GGT ATC GAG TAG GGT TAG GTG GG	I
YPO2088 S	*Y. pestis*	*ypo2088*	GAG TAG GGT TAG GTG GGC ATC A	I
YPO2088 A	*Y. pestis*	*ypo2088*	TTC AAC GCT TCA TTT TGT TGA CC	I
YPO2088 R	*Y. pestis*	*ypo2088*	GGC AAC AGC TCA ACA CCT TT	I
Y. pseu F	*Y. pestis/ Y. pstb*	*wzz*	AGA AGA YGG TTT RGA TAA AMG AGC GT	I
Y. pseu S	*Y. pestis/ Y. pstb*	*wzz*	AAC YGA GGG TGA MAA TGA ATA TCG CT	I
Y. pseu A	*Y. pestis/ Y. pstb*	*wzz*	GGA AAA CAT CAG CAT TAA CGA TGG TA	I
Y. pseu R	*Y. pestis/ Y. pstb*	*wzz*	GGA AAC ATC AGC ATT AAC GAT GG	I
Lambda F	λ Phage		ATG CCA CGT AAG CGA AAC A	I
Lambda R	λ Phage		GCA TAA ACG AAG CAG TCG AGT	I
toxR forward	*V. cholerae*	*toxR*	TGG CAT CGT TAG GGT TAG CAA	II
toxR reverse	*V. cholerae*	*toxR*	CAT TCA CAG CCC TGA AGT TTC A	II
ctxA forward	*V. cholerae*	*ctxA*	ACT CAC TCT GTC CTC TTG GCA TAA	II
ctxA reverse	*V. cholerae*	*ctxA*	GCA GAT TCT AGA CCT CCT GAT GAA AT	II
IS711 forward	*Brucella* spp.	IS*711*	GGC CTA CCG CTG CGA AT	III
IS711 reverse	*Brucella* spp.	IS*711*	TTG CGG ACA GTC ACC ATA ATG	III
cry forward	*B. thuringiensis*	*cry*[a]	GCT TCT CCT GTC GGT TTT TCG	III
cry reverse	*B. thuringiensis*	*cry*[a]	TGC ATT TCC CAT GGT TCC A	III
cryT forward	*B. thuringiensis*	*cryT*[b]	ATG GCT TCT CCT GTA GGG TTT TC	III
cryT reverse	*B. thuringiensis*	*cryT*[b]	GCT GCA TTT CCC ATA GTT CCA	III
cap forward	*B. anthracis*	*cap*	TTG GGA ACG TGT GGA TGA TTT	III
cap reverse	*B. anthracis*	*cap*	TCA GGG CGG CAA TTC ATA AT	III
pag forward	*B. anthracis*	*pag*	CGG ATA GCG GCG GTT AAT	III
pag reverse	*B. anthracis*	*pag*	CAA ATG CTA TTT TAA GGG CTT CTT TT	III
23 kDa forward	*F. tularensis*	*23 kDa*	TGA GAT GAT AAC AAG ACA ACA GGT AAC A	III
23 kDa reverse	*F. tularensis*	*23 kDa*	GGA TGA GAT CCT ATA CAT GCA GTA GGA	III
pla forward	*Y. pestis*	*pla*	GAA AGG AGT GCG GGT AAT AGG TT	III
pla reverse	*Y. pestis*	*pla*	CCT GCA AGT CCA ATA TAT GGC ATA	III

[a]Field test; based on NBCI databank accession number U87397
[b]Optimized assay based on the partially sequenced *cry* of the TUREX insecticide powder.

Table 13. Probes used in this study

Probe	Target organism	Target gene	Sequence (5' -> 3')	Reference
Y. pestis TM	*Y. pestis*	*pla*	6FAM-ACA GCA GGA TAT CAG GAA ACA CGT TTC AGT-BHQ1	I
Y. pestis TM	*Y. pestis*	*pla*	JOE-ACA GCA GGA TAT CAG GAA ACA CGT TTC AGT-BHQ1	I
YPO2088 TM	*Y. pestis*	*ypo2088*	ROX-TCC ATT TCA TGG CGG TAA TAT CGG GA-BHQ2	I
YPO2088 TM	*Y. pestis*	*ypo2088*	FAM-TCC ATT TCA TGG CGG TAA TAT CGG GA-BHQ2	I
Y. pseu TM	*Y. pestis / Y. pstb*	*wzz*	CY5-CAA CAA GTC ACG AGC RTC TGT CGG TGT-BHQ3	I
Lam TM2	λ Phage		YAK-ACC TTA CCG AAA TCG GTA CGG ATA CCG-DB	I
Lam TM2	λ Phage		ROX- ACC TTA CCG AAA TCG GTA CGG ATA CCG-DB	I
toxR probe	*V. cholerae*	*toxR*	FAM-CGT AAG GTT ATG TTT TCC-MGBNFQ	II
ctxA probe	*V. cholerae*	*ctxA*	FAM-ACC ACC TGA CTG CTT-MGBNFQ	II
IS711 probe	*Brucella* spp.	IS*711*	FAM-AAG CCA ACA CCC GGC-MGBNFQ	III
cry^a probe	*B. thuringiensis*	*cry*^{a}	FAM-CCA GAA TTC ACG TTT CC-MGBNFQ	III
cryT^a probe	*B. thuringiensis*	*cryT*^{a}	CCA GAA TTC ACT TTT CCG CT-MGBNFQ	III
cap probe	*B. anthracis*	*cap*	FAM-TAG TAA TCT AGC TCC AAT TGT-MGBNFQ	III
pag probe	*B. anthracis*	*pag*	FAM-TAG AAA CGA CTA AAC CGG ATA T-MGBNFQ	III
23 kDa probe	*F. tularensis*	*23 kDa*	FAM-CCA TTC ATG TGA GAA CTG-MGBNFQ	III
pla probe	*Y. pestis*	*pla*	FAM-TAA CCA GCG CTT TTC-MGBNFQ	III

6FAM = 6-Carboxyfluorescein, BHQ = Black hole quencher, YAK = Yakima yellow, DB = dabsyl, MGBNFQ = minor groove binder- nonfluorescent quencher

4.4 Optimisation of the assays and PCR conditions

The conventional PCR used in study I was performed using a Gradient Cycler (Eppendorf, Hamburg, Germany)and a temperature gradient from 58 °C to 69.5 °C for the annealing step in the temperature optimization assays. The correct size of amplicons was confirmed by traditional agarose gel electrophoresis in 2% agarose gels. The rtPCR was performed with a Rotorgene 2000 (Qiagen).

In study I, two sets of forward and reverse primers were designed for each target and first tested in a temperature gradient. The amplicons were analysed using agarose gel electrophoresis and estimated to be ca. 200 bp. The primer pairs giving one distinct and strong band in the gel were subsequently tested in a matrix with concentrations ranging from 50 to 900 nM. Probes were used at a concentration of 150 mM. The optimum combination of primers was selected based on the cycle threshold (Ct) value and the highest increase in fluorescence for the rtPCR detection. The rtPCRs were analysed directly in the Rotorgene 2000 using the analytical software provided with the instrument.

The probes in studies II and III were based on TaqMan technology developed by Applied Biosystems, with 5' nuclease chemistry and a sequence-specific dual-labelled hydrolysis probe that is cleaved off by the exonuclease activity of the Taq DNA polymerase. Optimal concentrations for primers and probes for the *V. cholerae*, *Brucella* spp. and *B. thuringiensis* assays were determined based on the lowest Ct-value and the highest increase in fluorescence signal. Primers and probes for *B. anthracis (cap* and *pag), F. tularensis (23 kDa)* and *Y. pestis* (*pla*) in study III were the same as previously reported (207). After re-evaluating the primer

concentrations in a test matrix, no changes were made to those previously used in Applied Biosystems (Foster City, CA, US) instruments. However, concentrations had to be slightly modified for use with the portable RAZOR instrument, which uses a four-fold larger reaction volume.

In studies II and III, a proprietary (Applied Biosystems, ABI) master-mix and appropriate volumes of primer and probe solutions were used and PCR amplifications were performed on the Applied Biosystems 7300 (Figure 7), 7500 and/or 7900HT Fast instruments. The RAZOR instrument (Figure 7) used in studies II and III could only be programmed to have three steps with different temperatures and a maximum of 4 min per step of the thermocycle. The initial 10-minute denaturation step used with ABI platforms was therefore not applicable when transferring assays to the RAZOR instrument and, therefore, the PCR programme had to be redesigned. The use of the Takara Premix Ex Taq polymerase (Takara Premix Ex Taq; Takara, Shiga, Japan), allowed the initial denaturation phase to be shortened to 10 seconds and the cycle denaturation phase (94 °C) to 5 seconds, meaning that the PCR run time could be shortened to approximately 40 minutes. In way of comparison, the ABI 7300 instrument run took 100 minutes and the ABI 7900HT Fast instrument completed the run in 35 minutes.

4.5 Testing analytical sensitivity and specificity

Analytical sensitivity (i.e., limit of detection) and specificity of the 4-plex *Y.pestis*/*Y.pseudotuberculosis* assay was determined using 10-fold serial dilutions of corresponding DNA as template. Primer specificity and the melting points of the amplified products were determined by a PCR-mix (SYBR®Green PCR Master mix, Applied Biosystems, Foster City, CA, US) containing SYBR Green and confirmed by sequencing. Analytical specificity was tested using the bacterial panel in Table 11. Approximate 1 ng of purified genomic DNA for each of the tested strains was used as template. Synthetic bacteriophage λ DNA was added as an internal amplification control, and DNA from *Y. pestis* strains EV76 and *Y. pseudotuberculosis* serotypes O:1b and O:3 as positive controls for *YPO2088/pla* and *wzz* assays, respectively. DNA extracted from *Pseudomonas aeruginosa* was used as a negative control and sterile water as a non-template control.

In studies II and III, the analytical sensitivities (limit of detection) of the assays were estimated with ABI and RAZOR instruments using 10-fold serial dilutions of the corresponding DNA as a template. Finally, sensitivity was confirmed with ten parallel reactions with 100 fg and 10 fg of template DNA per reaction. Analytical sensitivity was confirmed by comparing at least six parallel reactions of two separate 10-fold dilution series from 10^{-4} to 10^{-10} with the ABI instruments and four parallel reactions with the RAZOR instrument. The specificities were tested using the bacteria panel in Table 11 and 1 ng of purified genomic DNA from each of the tested strains as template. All PCR runs included a positive control of appropriate DNA, a negative (non-template) control and an internal positive control (IPC).

4.6 Testing the validity of with simulated samples

4.6.1 Spiked clinical samples (Study I)

Stratagene Mx3000P (Stratagene, La Jolla, CA, USA) and the Quantitect Multiplex PCR NoROX Kit (Qiagen) was used to validate the 4-plex *Y.pestis*/*Y.pseudotuberculosis* assay in study I for diagnostic purposes with spiked clinical samples. Sensitivity was determined using a dilution series of *Y. pestis* strain EV76. DNA was extracted by boiling from a 10-fold dilution series containing from 6000 to 1 bacteria in each reaction, and the boil-extracted DNA was used as a template. In addition, a pool of clinical sputum samples was prepared and spiked with a 10-fold dilution series of *Y. pestis* EV76 from 10^{-1} to 10^{-5}, which was added in a ratio of 1:10 to the sputum pool. DNA was then extracted from the spiked sputum pool using the MagnaPure LC System.

For specificity validation of the multiplex assay, and for detecting any possible inhibition caused, DNA was isolated from clinical human samples, such as swab samples from outer ear, throat, urethra, and chronic wounds which contained a rich and normal bacterial flora. Additionally, DNA from 80 clinical stool samples was purified automatically using the MagnaPure LC System, and analysed with the multiplex assay. The rtPCR run included synthetic bacteriophage λ DNA as internal amplification control, and DNA from *Y. pestis* strains EV76 and KIM D27 and *Y. pseudotuberculosis* serotypes O:1b and O:3 as positive controls for *pla, ypo2088* and *wzz* assays, respectively. Sterile water was used as a non-template control.

4.6.2 Spiked environmental samples (Study II)

The *V. cholerae* assay in Study II was tested with spiked environmental samples. *V. cholerae* (VC 243) was cultured in TS broth o/n and a 10-fold dilution series was prepared in 0.9% saline and in parallel to a previously autoclaved brackish seawater sample collected from the coast of the Gulf of Finland. The concentration of bacteria in the spiked samples was determined by plating a 10-fold dilution series in duplicate and determining the colony forming units / ml. The supernatants were used for PCR after inactivation with Proteinase K and centrifugation.

4.6.3 Spiked powder samples (Study III)

The total number of *B. thuringiensis* spores in the commercial insecticide (TUREX 50 WP, Certis, Columbia, MD, US) was estimated in a microscopy-counting chamber (Burker-Turk, Marienfeld, Germany) and the number of viable spores estimated by plating 10-fold serial dilutions of the insecticidal preparate. For the purpose of field-testing, the sample material was prepared by mixing the TUREX powder with rye flour at a ratio of 1:10 or 1:100. The negative control was unspiked rye flour. These three blind-coded training samples were transported to a mobile field laboratory with BSL-3 facilities, where the samples were

processed according to the standard operating procedures including filtration steps inside a class III and class II MSC. The filtrate was used as a template for the rtPCR analyses with the training *cry* assay. In order to detect any carry-over contaminants from sample preparation, a negative sample containing only PBS was included in the sample preparation work-flow.

Figure 7. Upper left: Applied Biosystems 7300 instrument in stationery laboratory. Upper right, Lower left and right: The RAZOR instrument can be used either in a research laboratory connected to a computer, when the fluorescence curve can be visually interpreted, or as a stand alone instrument with positive results shown with "+"sign and negative results as "-" signs on the instrument screen.

4.7. Performance evaluation study (Study IV)

4.7.1. Clinical samples and setting

A total of 1160 clinical faecal samples were prospectively collected into conventional sample containers or FecalSwabs™ from patients suspected of having *C. difficile* infection. The samples were analysed with the Orion GenRead *C. difficile* test (the index test) and comparative methods routinely used in laboratories participating in the study. Samples were analysed between 0 to 26 days after collection and, if not analysed immediately, were stored in a refrigerator. The three hospital laboratories participating in the study were Nordlab Oulu (Oulu, Finland), Fimlab Laboratories (Tampere, Finland), and Grenoble University Hospital Centre (CHU) (Grenoble, France). Our group at the R&D laboratory of Orion Diagnostica (Espoo, Finland) coordinated the study. Comparative methods used in this study were Illumigene *C. difficile* (Meridian Bioscience Inc, Cincinnati, OH, US) in Nordlab, and IMDx™ *C. difficile* for Abbott m2000 (Abbott Laboratories, Chicago, IL, US) in Fimlab. A two-step algorithm was used as a comparative method in Grenoble CHU, where faecal samples were first screened with the C. DIFF QUIK CHEK® test (TECHLAB, Blacksburg, VA, US) after which all GDH positive samples were confirmed by toxigenic *C. difficile* testing with the Xpert *C. difficile* assay (Cepheid, Sunnyvale, CA, US).

4.7.2 Reproducibility samples and setting

The reproducibility study of the index test was carried out with a panel of five samples. *Clostridium difficile* ATCC 43255 cells were spiked in an artificial sample matrix. The positive sample contained $5x10^5$ CFU/ml, the medium positive $8x10^4$ CFU/ml and the low positive sample $4x10^4$ CFU/ml of *C. difficile*. One sample contained *C. sordellii,* a close relative to *C. difficile* with similar toxin gene*,* in the amount of $2x10^5$ CFU/ml, and a negative sample containing only the artificial sample matrix. Samples were prepared at the Orion Diagnostica R&D laboratory and divided into three identical blind-coded panels that were distributed to the three participating laboratories (Nordlab, Fimlab, and the R&D laboratory at Orion Diagnostica).

4.7.3. Orion GenRead C. difficile test method

The Orion GenRead *C. difficile* test is a qualitative one based on an isothermal nucleic acid amplification technology (iNAT), SIBA (339). It is the first *in-vitro* diagnostic (IVD) test developed using this new technology and was used as the index test in this study. The target of the test is a conserved region in the *C. difficile* toxin B gene (*tcdB)*. The test was performed as instructed by the manufacturer, and the amplification reactions were run in the Orion GenRead instrument for 50 minutes. Temperature in the instruments stays at a constant of 41 °C. Results were automatically reported on the instrument screen as positive, negative or invalid. The positive reactions were reported as soon as the reaction reached the level of positivity as determined by the instrument software algorithm. In a case of an invalid result, a

new sample preparation was conducted as per the manufacturer's protocol and the run was repeated.

4.7.4 Comparative methods

Three different comparative methods were used in this study, each being routinely employed in the participating laboratories. The Illumigene *C. difficile* test (Meridian Bioscience Inc.) was used as a comparative method in Nordlab, Oulu and is a qualitative test based on isothermal loop-mediated amplification technology (LAMP). The target of the test is toxin A gene (*tcdA)* on the PaLoc region of the *C. difficile* genome. The sensitivity of the test has been previously reported to be 82.4-91%, and specificity 91-100% when compared with toxigenic culture (340–342). When compared to a rtPCR test (Amplivue, Quidel, San Diego, CA, US) the sensitivity was somewhat better; 96.1% and specifity of 99.8% (343). The sample was treated as per the manufacturer's instructions after which test and control tubes were placed in the Illumipro-10. This instrument reported results automatically as positive, negative or invalid. In case of an invalid result, the sample was re-run with less sample material as instructed by the manufacturer.

The IMDx *C. difficile* assay for the Abbott m2000 is an *in-vitro* rtPCR assay for the qualitative detection of toxigenic *C. difficile,* and it targets conserved regions in the *tcdA* and *tcdB* genes. It was used as the comparative method in Fimlab, Tampere. The sensitivity of the test has been previously reported to be 84.3%, and specificity 94.4% when compared with toxigenic culture (340). When compared with another molecular test with toxigenic culture performed only for for the discrepant results, sensitivity was reported to be 92.8% and specifity 100% (344). The test was performed according to the manufacturer's instructions and results were reported by the system as positive, negative or invalid. In response to an invalid result, the sample was re-run and cultured on *C. difficile* selective CCFA (Cycloserine Cefoxitin Fructose Agar, Oxoid Limited, UK) medium to observe *C. difficile* growth.

The C. DIFF QUIK CHEK® test (TECHLAB inc, Blacksburg, VA, US) is a rapid membrane-based enzyme immunoassay to detect GDH and often used as a screening test. The test does not distinguish toxigenic *C. difficile* strains from non-toxigenic ones. The test was used as a screening method at CHU Grenoble according to the manufacturer's instructions. The GDH positive samples were then analysed with the Xpert *C. difficile* assay, which is a qualitative rtPCR test for the identification of *C. difficile* toxin B and binary toxin from faecal samples. The sensitivity of the Xpert *C. difficile* test has been previously reported to be between 90-100% and specificity 97.9-100% when compared with toxigenic culture or other NAT tests (340,342,345). In one study, results from the two-step algorithm with C. DIFF QUIK CHEK and Xpert *C. difficile* were identical to the Xpert *C. difficile* test alone with sensitivity and specificity of 94.3% and 100%, respectively. The Xpert testing was performed as instructed by the manufacturer on an automated Cepheid GeneXpert® real-time PCR System (Cepheid, Sunnyvale, CA, US). After vortexing, the sample solution was transferred into a test cartridge

for the *C. difficile* assay program. Results were reported as positive, negative or invalid. The Xpert *C. difficile* assay was used in CHU Grenoble and also in Nordlab Oulu as a confirmatory method for discrepant results.

4.7.5 Controls and calculation of results

Each Orion GenRead test-run included a negative and a positive control for all study sites. A clean sample swab was used as a negative control and a swab sample taken from a pure culture of a toxigenic *C. difficile* was used as a positive control. In case of failure of either positive or negative control, the whole test series was rejected and the analysis was repeated. A pure culture of the *C. difficile* strain ATCC 9689 was used at Fimlab and Nordlab as a positive control, and in Grenoble CHU a clinical strain isolated from a faecal sample and confirmed with the Xpert *C. difficile* test was used. Discrepant results were defined as those having different results from the index test and the local comparative method. They were resolved by using a third either bacterial culture or another real-time PCR for toxigenic *C. difficile*. The sensitivity, specificity, overall agreement values for the Orion GenRead were calculated using the common 2x2 table format (Figure 8) according to approved methods (346,347).

		Comparative test	
		Positive	Negative
Index test	Positive	*number of true positive results (TP)*	*number of false positive results (FP)*
	Negative	*number of false negative results (FN)*	*number of true negative results (TN)*

overall percent agreement = 100% x (TP +TN)/(TP+FP+FN+TN)

positive percent agreement or estimated sensitivity = 100% x TP/(TP+FN)

negative percent agreement or estimated specificity = 100% x TN/(FP +TN)

Figure 8. The common 2x2 for reporting results when comparing the index test to comparative test and the formula for calculating the overall percent agreement, positive percent agreement or estimated sensitivity and the negative percent agreement or estimated specificity. TP = number of true positive results, FP = number of false positive results, TN = number of true negative results, FN = number of false negative results.

Methods used in this study are summarised in Table 14 below.

Table 14. Methods used in this study.

Method	**Used and described in**
Bacterial culture	I, II, III, IV
DNA isolation	I, III
Clustal W sequence alignment	I
Conventional PCR	I
Real-time PCR	I, II, III, IV
Melting temperature analyses	I
DNA sequencing	I, III
Agarose gel electrophoresis	I
Clinical sample preparation	I, IV
Cloning	III
Powder sample preparation	III
Spiked environmental sample preparation	II
Strand invasion based amplification (SIBA)	IV
Membrane-based enzyme immunoassay	IV

5. RESULTS AND DISCUSSION

5.1 Identification of BWAs and other pathogenic bacteria by real-time PCR (Studies I, II and III)

The aim of these studies was to develop detection and identification methods for rapid and accurate detection of biothreat agents and other pathogenic bacteria from diverse sample types using molecular techniques. Several novel real-time PCR assays were developed and tested with a panel of microbes. In these studies, we demonstrated the sensitivity and specificity of the developed assays with several sample types, including isolated DNA from relevant bacterial species and strains as well as spiked and simulated samples. We developed assays for biothreat agents that can be utilised for analysing samples taken from a suspected biothreat incident, from the environment, or samples of clinical origin. In Study I we developed a 4-plex assay for the simultaneous detection of *Y. pestis* and *Y.pseudutubeculosis*. The multiplex assay included two targets for the specific detection of *Y.pestis* and one target for *Y. pestis*/*Y. pseudotuberculosis*. In addition, an internal amplification control was included in the 4-plex assays. These assays can be used together in multiplex or as individual assays. In Study II we developed two real-time assays to *V. cholerae* from environmental samples. One assay successfully detectes regulatary gene *toxR* in all strains tested, while the second targets the *ctxA* gene in enteropathogenic strains. In Study III an assay was developed to detect all species of *Brucella* and another that detected a strain of *B. thuringiensis* in a commercial bioinsecticide. The latter was developed for field training simulation for the evaluation of powder agents (e.g., *B. anthracis* powder) that have been used by bioterrorists in the past.

The assays developed here are suitable for field use and fulfil the ideal criteria of being robust, lightweight and easy-to-use while still maintaining the decisiveness and quality of laboratory-based instruments. The second aim of our study was to evaluate a number of field-deployable platforms that would be fast and simple to use in resource-limited settings, outside centralised laboratories, in the field or in a developing country. We have successfully transferred several of the developed assays to field-deployable instruments. In study II, the *V. cholerae* assays were tested with three different platforms, one of which was a small and robust rtPCR instrument, RAZOR. In Study III, we transferred previously developed assays for *B. anthracis*, *F. tularensis* and *Y. pestis* to the RAZOR system as well as the newly-developed *Brucella* assay. The *B. thuringiensis* powder assay was tested in the Finnish Mobile CBRN Field Laboratory with a simple sample preparation using the RAZOR system. All of these assays were suitable for use in this system. It proved to be simple to use and provided rapid and reliable results in field settings with comparable performance as laboratory-based instruments. While the ABI 7300 has a larger capacity and has been successfully used in our field laboratory, the ABI Fast7900HT has delicate laser excitation technology that is not suitable for use in those conditions.

In Study IV we evaluated Orion GenRead, a novel isothermal platform in a multinational multicentre study with 1160 patent samples. Results demonstrate the utility and accuracy of this small, portable and light-weight "near-POC" instrument in a clinical laboratory setting. The Orion GenRead instrument can also be used as a stand-alone instrument without the need for a dedicated computer and can run in a battery-powered mode. Therefore, it is suitable for use in resource-limited settings, where the electrical currency can fluctuate. In this platform, there is no need for separate DNA isolation or purification step before the analysis, but the faecal sample is pre-treated in a simple few-step process before the analysis.. Assay reagents are freeze-dried in reagent tubes to maximize their portability, longevity and stability in fluctuating temperatures. Reagents also include an internal amplification control to detect their malfunction or that of the instrument. The positive results of the *C. difficile* test are shown on the instrument screen even as fast as in 15 minutes from the start of the run, and with the simple sample preparation the TAT can be less then an hour. This simple, fast and easy to use platform can help medical personnel make informed decisions concerning patient treatment and disease containment as soon as possible.

5.2 Multiplex real-time detection of *Y. pestis* / *Y. pseudotuberculosis* (Study I)

According to the CDC, *Y. pestis* is a category A biothreat agent and is highly contagious in an aerosol form. Following a deliberate release of *Y. pestis* in aerosolized form, rapid identification is crucial due to rapid progression of the possibly lethal pulmonary plague that will follow after inhaling the pathogen and for controlling the spread of the disease. Traditional methods of identification including bacterial culture, serology, and biochemical testing may take several days, all the while posing a risk of infection to laboratory personnel. Incidental aerosolisation may occur during sample preparation and strict adherence to PPE and MSC is paramount when handling clinical patient samples or samples suspected of containing BT agents. Using molecular methods to screen samples and identify pathogens diminishes the threat to laboratory personnel.

In study I, a multiplexed real-time PCR test was designed for the detection of *Y. pestis* containing a highly sensitive *pla* target situated in a plasmid, and a chromosomal target (*ypo2088*) unique to *Y. pestis.* While the *ypo2088* target is a reliable signature sequence for most *Y. pestis* strains, it was later discovered that there are strains lacking this target region. After designing of the *ypo2088* assay, genomic sequence of *Y. pestis* be. *antiqua* was added to GenBank (accession number CP000308) that does not contain the *ypo2088* target (32). This demonstrates how assays must continually be reviewed and updated in order to account for new variants of pathogens that would otherwise be missed. Fortunately in our case, the 4-plex assay contains two other targets for *Y. pestis* and would have detected the variants missing the *ypo2088* target. In addition to the *pla* and *ypo2088* targets we added the *wzz* gene; part of the lipopolysaccharide O-antigen gene cluster and a specific target for *Y. pestis* and its close relative *Y. pseudotuberculosis*. Amplification of *wzz* confirms either or both species of *Yersinia* are present, and amplification of *pla* and *ypo2088* will confirm the presence of *Y. pestis*. In

Finland and elsewhere in Europe, serotypes O:1 and O:3 are the most common causes of human epidemics caused by *Y. pseudotuberculosis* from various food sources, while in the Far East, serotypes O:4 and O:5 are more prevalent (348). Serotypes O:6–O:14 have not been isolated from human infections (349), and it was considered safe to exclude two most divergent serotypes with respect to the annealing site during assay design in the clinical context.

This study demonstrated that the developed assays worked well individually and when multiplexed, and were sufficiently sensitive and specific when used together with an internal amplification control for bacteriophage lambda DNA. Amplicon melting point analyses for the *Y. pestis* / *Y. pseudotuberculosis* multiplex assays revealed only one PCR product for each primer pair. The specificity of all amplicons was confirmed by sequencing.

Selected target sequences (*pla* and *ypo2088*) were specific to *Y. pestis* when tested with the isolated DNA from two different *Y. pestis* strains, 21 serotypes of *Y. pseudotuberculosis*, seven other *Yersinia* species, as well as eight other bacteria. No false-positive or false-negative results were observed using this panel. Specificity of the *wzz* gene as a marker of *Y. pestis* and *Y. pseudotuberculosis* in PCR using a different alternate primer pair was validated with 259 bacterial strains without any false positive or negative results (322). The combined results of this work, including the analysis of 80 stool samples allowed us to come to the conclusion that the *wzz*-based PCR is specific for the detection of *Y. pestis* and *Y. pseudotuberculosis*.

Lower detection limits for individual *Y. pestis* assays were 10 fg of purified bacterial DNA for the *pla* assay and 100 fg for the *ypo2088* assay. Detection limits in the 4-plex assay were determined to be 1 CFU for both the *pla* and *ypo2088* assays according to a 10-fold dilution series of *Y. pestis* strain EV76. No false positives or false negatives were obtained with either assay for *Y. pestis* when challenged with related and unrelated bacteria described in Table 10. The *Y. pestis* assays did not react with any of the *Y. pseudotuberculosis* O-serotype strains. The *wzz* assay was able to detect all tested O-serotypes, except for the O:6 and O:7 that could not be incorporated to the assay design due to base mismatches in the annealing site.

The utility of multiplexed *Y. pestis/Y. pseudotuberculosis* assays for the detection of *Y. pestis* in a clinical sample matrix was shown using pooled sputum samples into which a dilution series of *Y. pestis* EV76 strain cells was spiked. When the total DNA was extracted from the spiked samples and used for PCR, assays were able to correctly detect the *Y. pestis* targets. In a further specificity study with 80 clinical stool samples containing a rich microbiota, total DNA extracted did not induce any false positive reactions in the *Y. pestis/Y. pseudotuberculosis* assays. When spiked, both target bacteria were correctly detected in these samples and neither of the sample matrices showed any inhibitory effects on assays.

Assays could be used separately, if so desired, but the multiplexed assay contains several markers for *Y. pestis* in one PCR reaction and increases reliability while accounting for strains

lacking the *pPla* or *ypo2088* target. Therefore, the multiplex PCR assay described here is suitable for the screening of clinical patient samples during a suspected release or outbreak of *Y. pestis* or *Y. pseudotuberculosis*. Although not tested with other types of sample matrix, it could be envisioned that after DNA extraction the multiplexed assay would work with other types of sample matrix as well such as swabs, environmental or food samples. The stool sample matrix is undoubtedly one of the most difficult to work with since it contains many PCR inhibitors. As such, it seems safe to assume that other sample matrices would work as well following appropriate sample preparation, but this should be investigated fully before putting into practice.

The *wzz* assay may also be valuable as a stand-alone test in clinical diagnostics. In Finland, sporadic food-borne infections and outbreaks of *Y. pseudotuberculosis* have occurred thus a reliable and sensitive detection method is valuable. Diagnostic systems for *Y. pestis* are needed to screen international travellers originating or passing through high-risk zones, especially in the light of the recent refugee crisis in the Middle East and Europe. Infections could also be caused by a deliberate release of the pathogen in a bioterrorism event, for which governments need to be prepared.

5.3 Multiplatform real-time PCR assay for *V. cholerae* (Study II)

Vibrio cholerae is a common inhabitant of aquatic environments in Asia, Africa, Latin America and the Caribbean. The bacterium is a significant pathogen in developing countries where sanitation and a potable water supply are limited, and is also considered to be a potential biothreat agent. As such, a fast, reliable, preferably field-deployable assay is needed for its detection and identification. In study II, we successfully developed assays for the detection of both non-pathogenic and the enterotoxigenic O1 and O139 strains. Real-time PCR assays with TaqMan detection probes were easily transferred between two Applied Biosystems platforms and to the field-ready RAZOR system. RAZOR can generate reliable results in around 40 minutes making it especially well-suited to emergency situations. The ABI 7900HT Fast instrument was even faster with a run-time of 35 minutes, but these platforms are larger or rely on delicate technology that make them less suitable for field or mobile laboratory use. The ABI 7300 instrument has been used successfully in a mobile laboratory but it has a longer run time of 100 minutes making it inferior to the RAZOR system.

Assays for identifying enterotoxigenic *V. cholera* from other strains and species of *Vibrio* were based on genes encoding the ToxR activator (*toxR*) and enterotoxin A protein (*ctxA*) and tested on three platforms; ABI 7300, ABI 7900HT Fast and RAZOR. Analytical sensitivity of both assays was 100 fg of purified gDNA/reaction, which is equivalent to around 20 genomes. In its natural habitat (i.e., estuaries and coastal environments of Bangladesh and Peru), *V. cholerae* occurs in densities up to $5x10^3$ to $6x10^5$ cells/ml (350,351). Our aim was to develop assays that are sensitive enough to detect this natural density of *V. cholerae* cells in environmental water samples. The sensitivity with spiked saline and brackish water samples

was at least 1800 CFU/ml with the Applied Biosystems instrument and 180 CFU/ml with RAZOR, corresponding to 2 CFU/reaction in the ABI 7300 and RAZOR instrument and 5 CFU in the ABI 7900HT Fast machine. Our assays could detect presence of the bacterium at levels that were 3- to 3000-fold less than natural densities. Similar sensitivities have been shown earlier by Gubala and Proll and by Chomvarin et al. (255,352). Sensitivity of the RAZOR instrument was higher due to the larger sample volume per reaction (total volume of around 100 µl/ reaction). This indicates that the developed assays are suitable for field-testing of natural waters. Brief pre-treatment of spiked seawater samples showed no inhibition of PCR efficiency, which also indicates the utility of these assays for reliable identification of *V. cholerae* in environmental water samples.

Assays were challenged with 63 strains from various sources. The *toxR* assay correctly detected all strains which included non-pathogenic and pathogenic El Tor O1 strains. The *ctxA* assay correctly detected two El Tor O1 strains. Analytical specificity of the assays was further tested with a bacterial panel containing closely-related bacteria and other water-borne pathogens specified in Table 11. Neither of the assays gave false positive reactions when tested with this panel in any of the three platforms used. Four *Serratia marcescens* strains and a heat-labile enterotoxin producing *Escherichia coli* strain were also tested, since it was determined during the design phase that sequences of these organisms were highly similar to the assay target sequences. However, none of the *S. marcescens* or *E. coli* strains tested reacted with our assays. Based on this testing we conclude that analytical specificity of the developed rtPCR assays was sufficient for use.

Assays were easily transferred among the tested platforms and all showed similar sensitivity and specificity. The assay run time is dependent on the platform, the ABI 7900HT Fast had the fastest PCR run time of 35 minutes but, as mentioned above, laser technology in this instrument restricts its use to the laboratory bench. The field-ready RAZOR instrument performed as well as the larger and more delicate ABI platforms. In conjunction with the RAZOR platform, the developed assays could be used to detect *V. cholerae* in environmental samples in field or resource-limited settings. This makes a robust diagnostic system for cholera available to developing countries with poor infrastructure and a disperse population as well as medical personnel working in crisis situations.

5.4 Detection of *B. anthracis, Brucella* spp., *F. tularensis* and *Y. pestis* and comparison of platforms (Study III)

The CDC considers *B. anthracis, F. tularensis* and *Y. pestis* category A biothreat agents of the highest concern. *Brucella* spp. are considered category B due to less severe manifestation, low mortality and slow onset of the disease. Furthermore, its presence on the BT/BW agent list can be though of as a consequence of *Brucella* being studied as a BW agent in the past but one that is no longer considered to be an ideal agent. In study III, we successfully transferred the previously designed assays for three category A agents (i.e., *B. anthracis* [with two targets, *cap*

and *pag*], *F. tularensis* [*23kDa* assay] and *Y. pestis* [*pla* assay]) to a field-ready real-time PCR instrument (RAZOR) and compared the analytical sensitivity and specificity of the assays in the larger laboratory instruments (ABI 7300/7500). Our testing showed that there were no notable differences in the results generated by these platforms when purified genomic DNA was used as a template. Detection limits were similar to those reported earlier, 10-100 fg of gDNA/reaction (353), corresponding to 1-10 genomic copies/reaction.

During transfer of the assays to the RAZOR instrument, the PCR programme and reagents had to be modified to suit the new platform. The run time was already shorter in the RAZOR instrument, but it could be further shortened with the use of a "hot start" polymerase and master mix optimised for fast amplification. The total PCR run time after optimisation was 40 minutes on the RAZOR instrument, compared with the 100 minutes on the ABI 7300 and 7500 instruments.

Primers and probes targeting insertion sequence *IS711* were newly designed to detect all species of the genus *Brucella* for a rapid broad-range detection, possibly in the field or mobile laboratory, which can be followed by species identification elsewhere, if needed. The selected *IS711* target was shown to be specific for the genus *Brucella* by using a panel of *Brucella* species, related pathogenic bacteria, and other bacteria containing similar sequence to the target. Recently, several general and specific assays have been developed for the detection of *Brucella* species using *IS711* as a target (243). While assays are sensitive to this target, Bounaadja et al. demonstrated that the detection limit differs among species due to a variable number of target repeats in their genome. The limit of detection of our assay was identical in both real-time instruments tested (10 fg/reaction, approximately 2-3 bacteria/reaction) and which is similar or slightly lower than for previously developed assays (354).

Sensitivity for the assays of *B. anthracis-cap*, *F. tularensis* and *Y. pestis* were 10 fg of gDNA/reaction and for the *B. anthracis-pag* and the *B. thuringiensis* assay 100 fg of gDNA/reaction, as determined with a 10-fold dilution series of purified genomic DNA of the bacterial panel described in Table 11. These sensitivities were similar across the three platforms when purified DNA was used, and no false positive or negative reactions were observed with any of the assays or platforms tested.

These assays provide rapid tools for the specific detection of the targeted BT/BW agents. No notable differences between the ABI 7300/7500 instruments and the RAZOR instrument were observed in analytical sensitivity or specificity under optimal conditions in our study. However, RAZOR offers significantly shorter reaction times than other tested instruments (40 vs. 100 minutes) and is more robust, field-ready instrument. The system could be improved by developing simple sample preparation solutions for rapid testing of the BW/BT agents under field conditions.

5.5 Field-training assay for *B. thuringiensis* powder (Study III)

An assay was developed with which to train personnel in the screening of suspected BW/BT agents as powder samples in the mobile field laboratory. This assay, to our knowledge and during the time of our study, is the first of its type to be developed for a powder sample. We used powdery substance containing *B. thuringiensis* as a simulant agent for *B. anthracis* spores. Using the training assay and a crude two-step sample preparation protocol to screen for the presence of *B. thuringiensis* in a commercial insecticide powder, we correctly detected the agent in blind-coded samples in the mobile laboratory. After sequencing the target, the original assay based on *cryT* was shown to include a few mismatches in the annealing site, and the assay was re-designed accordingly. With this newly-optimised assay, the analytical detection limit was determine to be ca. 100 fg of purified DNA, which was adequate for the training purposes.

Observed differences between the sensitivities of the platforms tested favoured RAZOR, which was more sensitive when purified TUREX insecticide powder was used as a template. The reaction volume in the RAZOR instrument is four times higher (100 µl) than in the ABI 7300 instrument (25 µl), which may account for the superior performance of the reactions due to dilution of possible inhibitors. We did not observe cross-reactivity or false positive reactions with either of the instruments, even though we observed the amplification of the *cry* target with the primers containing the mismatched nucleotides. The RAZOR instrument in particular seemed to be more robust in this respect, showing a larger detection range with the mismatched primers. Similar to earlier trials, the RAZOR instrument provided a rapid tool for screening as the PCR reaction time with optimised reagents and program was only 40 minutes long and a larger sample volume may be more appropriate for the detection of BT/BW agents in field conditions, even if they contain slight changes or modifications in the target sequences. The ability to to sustain some mismatches is also dependent on the reagents and the proofreading characteristics of the polymerase used.

The simple sample preparation technique that we designed and tested in field conditions in this study bypassed the need to isolate target DNA from the TUREX powder sample, saving time and reducing complexity of testing in field conditions. No specific reagents or instrumentation, such as specific enzymes, centrifuges or heat blocks for the sample preparation were needed to prepare samples in the field. The developed procedure was simple, safe and an excellent system by which to provide safe training of mobile laboratory personnel for sampling and screening of suspect powders.

5.6. Performance of the Orion GenRead *C. difficile* test (Study IV)

Nucleic-acid based POC testing has recently become more popular and the trend has been towards more automated, simple-to-use, and smaller testing platforms. In study IV, we evaluated the accuracy and utility of a novel isothermal platform (Orion GenRead) for detecting toxigenic *C. difficile* in clinical settings. The Orion GenRead C. difficile test comprises

of isothermal reagents and is the first diagnostic test to use SIBA. The test is run on a small instrument that is lightweight and battery powered. The instrument contains a graphical user interface and can process 1-12 samples at a time in a single PCR program. The positive results of the Orion GenRead *C. difficile* test are shown automatically on the instrument touch screen when ready, within 15 minutes at best. The entire runtime of the *C. difficile* test is 50 minutes, after which results are shown in a clear and unambiguous form as positive or negative, thus aiding rapid patient treatment and management decisions. The assay also contains IAC that signify the presence of inhibitors. In case the target and IAC fail to amplify, the instrument will display an invalid result. This helps prevent false negative results due to, e.g., presence of inhibitors or reagent failure. In our study, invalid results were reported for less than 3% of reactions, and it could be concluded that a number were due to leakage of the test reagent tubes, which happened at the beginning of the study and was later corrected.

Out of the 1160 samples analysed in this study, 184 (15.9%) were considered to be true positives and 949 (81.8%) true negatives for toxigenic *C. difficile* (Figure 9). Eleven false positives (0.9%) and 16 false negatives (1.4%) were observed with the evaluated test system as compared to the test routinely used in participating hospital laboratories. Results are summarised in Table 15. A total of 45 invalid results were obtained with the Orion GenRead C. difficile test. These 45 were excluded from the performance evaluation calculations and samples were re-run according to manufacturer's instructions. Successful results from re-runs were added to the result calculation.

Table 15. Results of the performance evaluation against three different comparison methods and two sample collection methods.

Comparative method result		Orion GenRead C. difficile result		Total amount of samples tested	Estimated Sensitivity (%)	Estimated Specificity (%)	Overall agreement (%)
Illumigene *C. difficile*		*positive*	*negative*	397	91,9	98,1	96,7
	positive	79	7				
	negative	6	305				
IMDx *C. difficile*				170	92,0	100	98,8
	positive	23	2				
	negative	0	145				
IMDx *C. difficile**				437	91,9	99,7	98,4
	positive	68	6				
	negative	1	362				
C. DIFF QUICK CHECK + Xpert *C. difficile*				156	93,3	97,2	96,8
	positive	14	1				
	negative	4	137				

* Samples collected with Fecalswab®

In Nordlab Oulu where the Orion GenRead C. difficile test was compared to the Illumigene *C. difficile* test, a total of 397 faecal samples were analysed with both methods. Of these, 79 (19.9%) were regarded as true positives and 305 (75.8%) as true negatives in the Orion GenRead C. difficile test when compared to the Illumigene *C. difficile* test. Discrepant results were reconciled by using Xpert *C. difficile* rtPCR test and one sample was re-classified as true positive (positive with Orion GenRead and Xpert, also positive in bacterial culture after enrichment, negative with Illumigene) and one sample was classified as true negative

(negative with Orion GenRead and Xpert and in culture, positive with Illumigene). Seven samples were classified as false negative and six as false positive. The false negative rate was 1.7% and the false positive rate 1.5%. The overall agreement of the tests was 96.7%, and sensitivity of the Orion GenRead test was 91.9% and specificity 98.1% when compared with Illumigene. This is in the same sensitivity range as has been obtained earlier for the Illumigene *C. difficile* test, when it was compared with toxigenic culture, but when compared to rtPCR, the Illumigene test was slightly more sensitive (341-344).

Two different sample collection methods were included in the study conducted at Fimlab Tampere where 170 faecal samples collected in conventional sample containers and 437 faecal samples collected in FecalSwabs were analysed with the IMDx *C. difficile* and the Orion GenRead *C. difficile* tests. With respect to those collected in the conventional sample containers, the sensitivity and specificity of the Orion GenRead test were 92.0% and 100.0%, respectively. 23/170 (13.5%) samples were true positives and 145/170 (83.5%) true negatives. Two false negative results and no false positives were observed with the Orion GenRead test, giving a false negative rate of 1.2% and false positive rate of 0%. The overall agreement of the tests of collected in the conventional sample containers was 98.4%. With samples collected with FecalSwabs, the sensitivity and specificity were 91.9% and 99.7% and the overall agreement 98.8%. 68/437 (15.6%) of the samples were true positives and 362/437 (82.8%) true negatives. Six false negatives and one false positive result were obtained, giving a false negative rate of 1.4% and a false positive rate of 0.2%. The sensitivity and specificity obtained with the Orion GenRead *C. difficile* test was in range obtained previously with the IMDx *C. difficile* test. When compared with toxigenic culture, the sensitivity and specificity were IMDx test were lower. In one study, the sensitivity of the IMDx assay was only 61.1%, but specificity 99.4% (355). In this study, frozen samples were used for IMDx testing and it was concluded that IMDx could not detect *C. difficile* in samples with low bacterial load.

At Grenoble University Hospital, the routine method for *C. difficile* diagnosis is to first screen all the suspected faecal samples with antigen based C.DIFF QUIK CHEK test and then to confirm the positive result using Xpert *C. difficile* real-time PCR. All samples, both negative and positive, were tested with Orion GenRead. Unlike in Finland, where formed faecal samples are also tested, unformed faecal samples are sent to laboratories in France for toxigenic *C. difficile* diagnosis. The overall agreement among methods was 96.8% and the sensitivity and specificity values were 93.3% and 97.2%, respectively. This is within the range reported for the Xpert *C. difficile* test when compared to toxigenic culture or other molecular tests. 14/156 (8.9%) samples were true positives and 137/156 (87.8%) true negatives. 1/156 (0.6%) false negative and 4/156 (2.6%) false positive results were observed.

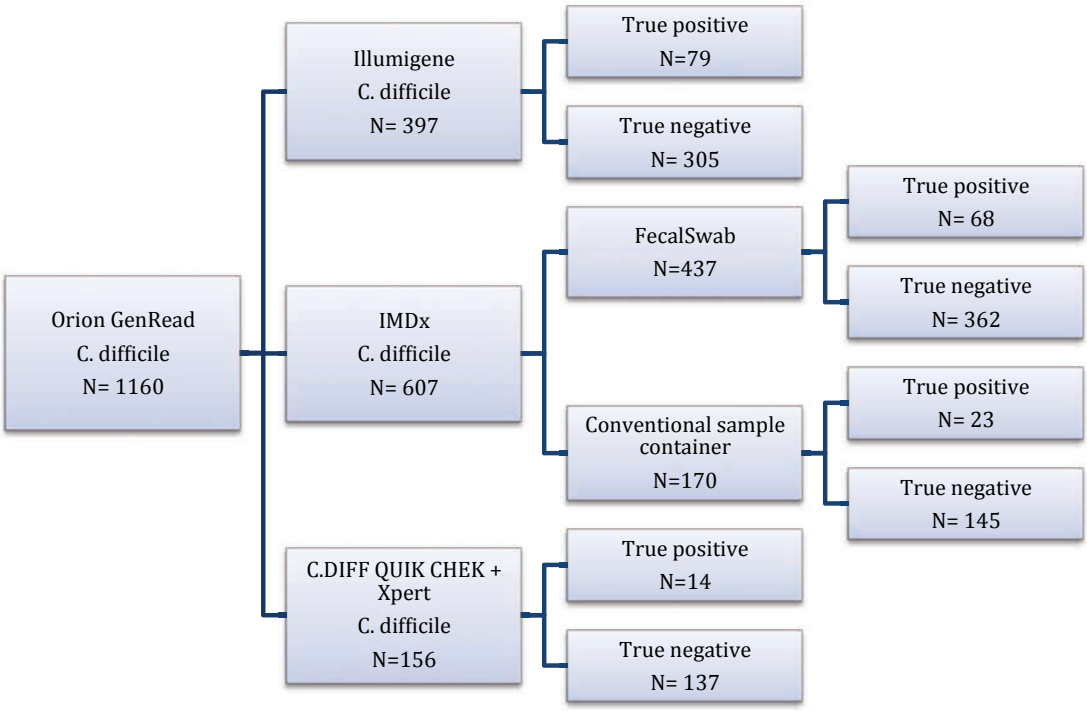

Figure 9. The number of samples tested with the Orion GenRead C. difficile test, compared to other methods showing breakdown of results obtained in the accuracy study. Samples were collected in conventional sample containers unless otherwise stated. A total of 45 invalid results were obtained for the 1160 samples analysed with the Orion GenRead *C. difficile* test and 47 with the 397 samples analysed with the Illumigene *C. difficile* test. With the IMDx test system, 5 invalid results were obtained for samples collected with FecalSwabs (N=437) and 1 with samples collected into conventional sample containers (N=170). When C.DIFF QUIK CHEK + Xpert *C. difficile* testing algorithm was used, no invalid result were obtained. N = number of samples tested

5.6.1 Reproducibility of the Orion GenRead C. difficile *test (Study IV)*

The same set of blind-coded samples was analysed in three participating laboratories in the reproducibility study, the samples containing a positive, medium positive, low positive and negative sample plus a sample with closely related *C. sordellii* cells. All the samples were analysed in three replicates, two runs per day by two different operators at each site for five days. In this study the total per cent agreement between the participating laboratories was 99.8%, with only one false positive result (Table 16).

Table 16. Reproducibility test results from participant laboratories.

	Orion Diagnostica R&D laboratory		Nordlab Oulu		Fimlab Tampere		Total	
Sample type*	**correct results/ total**	**Agreement (%)**	**correct results / total**	**Agreement (%)**	**correct results / total**	**Agreement (%)**	**correct results / total**	**Agreement (%)**
Negative	30/30	100	30/30	100	30/30	100	90/90	100
Low positive	30/30	100	30/30	100	30/30	100	90/90	100
Medium positive	30/30	100	30/30	100	30/30	100	90/90	100
Positive	30/30	100	30/30	100	30/30	100	90/90	100
C. sordellii	29/30	96,7	30/30	100	30/30	100	89/90	98,9
Total	149/150	99,3	150/150	100	150/150	100	449/450	99,8

*All samples were prepared to an artificial sample material containing 5% DMEM, 1% FCS (w/v) in commercial FECLONE product. The amount of *C. difficile* ATCC 43255 cells in the samples was $4x10^4$ cfu/ml in the low positive, $8x10^4$ cfu/ml in the medium positive and $5x10^5$ cfu/ml in the positive sample preparate. The amount of *C. sordellii* ATCC 199714 cells in the *C. sordellii* sample was $2x10^5$ cfu/ml. The one positive result obtained with the *C. sordellii* sample was due to an error in sample handling.

5.6.2 Conclusions of the Evaluation study (Study IV)

In our evaluation study, the Orion GenRead C. difficile assay was shown to have good analytical performance (overall agreement varying from 96.7 to 98.8 %) when it was compared with the three methods used in the participating hospital laboratories for the routine analysis of suspected CDI samples. The Orion GenRead C. difficile test had a similar sensitivity to other methods applied in earlier studies. Although it was not possible within the context of this study to employ a standard protocol across all participant laboratories, results were similar at all three sites. Using a blind-coded test panel at three sites in Finland during five consecutive working days, with two runs each day conducted by six different laboratory technicians, test reproducibility was 99.8%. In conclusion, the Orion GenRead system is a suitable screening system for toxigenic *C. difficile* in faecal samples and performs well in comparison to other tests routinely employed at two hospital laboratories in Finland and one in southern France.

The Orion GenRead C. difficile test could also be used for toxigenic *C. difficile* screening test in a two-step testing protocol followed by a test to confirm toxin production in a case of active CDI. The platform could even be adapted for use for high-throughput screening in a central laboratory because of its fast run time that enables four batches of 12 sample runs (48 samples) to be run in less than 3.5 hours, and 5 batches (72 samples) in less than 4.5 hours, with little requirement for space and power. This performance is comparable to a larger instrument such as the Abbott m2000 system which processes 1-48 samples in 3-4 hours and the 49-96 samples ca. 4-4.5 hours.

5.7 Benefits and precautions of POC testing

Rapid POC tests can assist healthcare professional by delivering rapid results and hasten the time taken to make an informed decision concerning patient treatment and disease management (11,44). Fast diagnostic results given by POC tests may also mean reduced total cost for patient care as the extra expense of the test is recovered by a more rapid recovery, requiring less medication of the correct type and limiting the spread of any disease at an early stage by appropriate isolation and lockdown practices. POC testing for infectious disease agents has the possibility to save many lives, especially in developing countries, by increasing access to proper diagnosis and therefore treatment (97). POC testing may also improve health care globally, when it is is carefully evaluated and correctly employed (82). However, whenever POC testing is employed in a field situation, it is prudent to have external support from a clinical microbiological laboratory or a clinician specialising in infectious diseases to ensure accuracy and the correct interpretation of patient status.

Isothermal technology, such as SIBA, could enable the construction of less sophisticated and more affordable instruments that do not require the precise control and rapid temperature adjustment of standard PCR thermal cyclers. The Orion GenRead platform (Figure 10) is a step towards more cost-effective molecular POC technologies that could be used in developing countries. This kind of POC test platform could also be adapted for detection of biothreat agents in field conditions. The ease of use, portability, and small footprint of the instrument are well suited for mobile laboratory use and the rather simple sample preparation method could be further optimised for selected agents. The simple format of the result readout is also suitable even for situations where little medical expertise or support is available.

Figure 10. The Orion GenRead C. difficile test system used in study IV includes a small portable instrument, freeze-dried isothermal DNA amplification reagents and materials necessary for faecal sample preparation.

6. SUMMARY AND CONCLUDING REMARKS

The main results of this study can be summarized as follows:

I The developed multiplexed real-time PCR assay for the detection and identification of *Y. pestis* and *Y. pseudotuberculosis* utilising *pla*, *ypo2088* and *wzz* targets is sensitive, specific and suitable for use with clinical samples.

II The *V. cholerae* assays utilizing *toxR* and *ctxA* gene targets can be used for sensitive, specific and rapid detection of non-pathogenic and pathogenic *V. cholerae* from environmental water samples using multiple platforms, such as the portable and field-deployable RAZOR system, or the Applied Biosystems 7300 or 7900HT Fast instruments.

III The assays for *B. anthracis*, *Brucella* spp., *F. tularensis* and *Y. pestis* can be used with the field-deployable RAZOR instrument as well as with the Applied Biosystems 7300/7500 instruments for rapid, sensitive and specific detection and identification of the BT/BW agents.

IV The newly developed field-training assay for the detection of *B. thuringiensis* in powder samples can be used with the simple sample preparation protocol developed for training purposes in the mobile field laboratory.

V The Orion GenRead C. difficile assay, which is based on isothermal amplification technology called SIBA, and run on a novel, small and portable instrument, is a fast and accurate method for detecting toxigenic *C. difficile* in faecal samples and can be used to confirm CDI diagnosis.

In the wake of a bioterrorist or bioweapon attack, it is critically important to detect and identify the biological agent(s) involved as soon as possible. An accurate, fast and reliable detection and diagnostic system ensures that informed decisions are made and appropriate measures are taken for the protection and treatment of the affected population. It also provides important information to government agencies during and after an attack to limit the impact and spread of an agent, respectively. It is of great advantage if the detection and identification can be performed directly in the field via a portable device or in a mobile field laboratory near the affected area, thus minimizing the time spent transporting samples to central laboratories. Fixed-location laboratories in the affected area may lack the expertise,

technology, training or required biosafety level laboratories to detect and diagnose the BT/BW agents involved. In a suspected attack involving biothreat agents, the first rapid detection that is performed in the field would most probably be followed with forensic sampling and more accurate identification such as sequencing and strain typing in an appropriate reference laboratory. This confirmation would then provide information relevant for international justice and court proceedings to determine if the release of biological agents was deliberate and if the BWC convention was violated.

In the public health sector, many of the large clinical microbiology labs have moved towards centralisation of molecular diagnostics with automated high-throughput instruments. In contrast, NAT-based POC diagnostics have been developed during recent years to be used in more decentralized or less developed settings (44,89,356). An increasing number of molecular POC instruments and platforms for the identification of multiple pathogens that are easy to use and require less expertise of the user are being developed. Development of POC technologies is being driven by similar criteria as the field detection of BT/BW agents. The ASSURED criteria of affordable, sensitive, specific, user-friendly, robust and rapid tests with the use of minimal equipment that were developed for POC testing, are appropriate for BT/BW agent detection as well. Developments in infectious disease diagnostics and clinical microbiology are directly applicable to BT/BW agents as these two fields are closely related and employ similar methods.

In the present study, we have demonstrated the development of sensitive and specific assays for the detection and identification of several bacterial biothreat agents from different sample types. These assays can be used in stationary laboratories but we have also demonstrated their utility in portable systems suitable for mobile laboratories and field situations. We successfully designed a training assay for powder samples and tested it in field conditions. Finally, we tested a novel and portable isothermal technology platform and demonstrated its utility for the identification of toxigenic *C. difficile* from clinical faecal samples in a multicentre, multinational accuracy study of 1160 samples. This platform could well be suited for use in a field laboratory or resource-limited setting due to its small footprint, ease of use, portability, optional battery power, and fast generation of decisive results. Sample preparation could be simplified and in an ideal case would be an automatic feature of the test system protocol. This is also true for any rtPCR methods that require DNA isolation and purification prior to thermal cycling.

Developments in molecular biology, next generation sequencing, and nanotechnology continue to make these systems ever faster, cheaper and more simple to use. These technologies will become more affordable and innovation will hasten the clinical diagnosis of microbial pathogens. Innovations that combine microfluid devices with isothermal amplification and smartphone readers for rapid detection of disease causing agents are currently in development and testing (9). Perhaps the most demanding issue remaining to be solved is the preparation of different sample types into a suitable form for use in microfluidic

or nanotechnology devices, and the discovery of analytical methods that require minimal or no sample preparation at all.

ACKNOWLEDGEMENTS

This work was carried out at the Department of Bacteriology and Immunology, Medicum, University of Helsinki, at HUSLAB, at the Centres for Military Medicine and for Biological Threat Preparedness, the Finnish Defence Forces, and at the Research and Development Department, Orion Diagnostica Oy.

I would like to express my deepest gratitude to my supervisors, Professor Mikael Skurnik, Professor Simo Nikkari and Docent Minna Mäki for their support, scientific expertise and all their precious advice during this work. I am grateful for Professor Mikael Skurnik, who first took me under his wing when I returned to Finland after working some years abroad, and skilfully guided me in taking my first steps in the field of molecular medicine. I appreciate his good sense of humour and relaxed, but always professional way of working. I thank Professor Simo Nikkari for the opportunity to work in the exciting field of military medicine and to join the EU Nordic Battle Group (NBG) to work with the most skilled professionals in the CBRN protection field. I also thank Simo for his encouragement and practical advice during the laboratory experiments and writing the manuscripts. Docent Minna Mäki is thanked for her professional guidance in the world of molecular POC diagnostics, and all her invaluable advice in planning, analysing and reporting the work of the fourth publication, and for her optimism and support in finalising this thesis. Her merry companionship during business trips and many scientific discussions in long evening hours of work are highly appreciated.

Professor Hannu Sarkkinen and Docent Matti Waris are warmly thanked for their time and expertise in reviewing this work, and for all valuable and constructive criticism that significantly improved this thesis. I am very honoured to have Doctor Vanya Gant from the University College London Hospital acting as my opponent in the thesis defence and thank him for finding time in his busy schedule to come to Helsinki.

No-one can do the work alone, and my warmest thanks and gratitude go to all my co-workers during these years at the University of Helsinki, HUSLAB, Finnish Defence Forces and Orion Diagnostica. You are the salt and sugar of life. Especially I want to thank Heidi Hemmilä and Heli Piiparinen, whom I worked with at the Centre for Military Medicine, for making the laboratory work always so smooth, and for all the wonderful field trips we took together. For always a pleasant company and supportative working atmosphere I would like to thank Ava Sovijärvi, Mika Aho and Ari Hörman. I also want to thank Paula Maatela and Tuuli Haataja from the Finnish Defence Research Agency (FDRA) for excellent co-operation with regards to the Field Laboratory, and the marvellous time we spent together at the Battle Group. Majors Heikki Salonen and Jukka Pääkkönen, head and deputy head of the CBRN Detachment, and Captain Katri Teho, head of SIBCRA patrol, and all my co-fighters of the Field Laboratory Platoon and the whole CBRN Detachment are thanked for the numerous memorable SIBCRA

and combat fighting training missions during the NBG assignment. The little over year spend with you guys in the Finnish forests was one of the best ones in my life, and gave me the best concrete and practical knowledge on work in the CBRN defence field.

I sincery thank the team in Orion Diagnostica R&D and beyond, Sanna Filén, Riikka Kärkkäinen, Tuula Siljander, Solveig Sjöblom, Tiina Töyrylä, Johanna Elgh, Anniina Wester, Kirsi Pousi, Päivi Rikkinen-Holm, Tuomas Ojalehto, Lahja Uitto, Ossi Korhonen, Mirko Brummer, Kevin Eboigbodin, Anssi Rantakari, and many others for always skilful and professional work, excellent everyday working collaboration, and all the great lunch hour discussions together, not to mention the wonderful parties and other off the work activies. The *Yersinia* group in Helsinki, Marta Biedzka-Sarek, Alexandra Götz, Tea Blom, Saara Salmenlinna, Chun-Mei Li and Anita Saarinen are thanked for good times, great discussions and all scientific and technical support during the laboratory work for my first paper.

All my co-authors earn many thanks for the fruitful collaboration, special thanks go to Katja Koskela, Heidi Hemmilä and Heli Piiparinen from the Centre of Military Medicine, Jari Hirvonen and Risto Vuento from Fimlab, Tanja Holma, Päivi Tissari and Martti Vaara from HUSLAB, Jaana Kauppila and Hanna Tuokko from Nordlab, and Sandrine Boisset from the University Hospital Laboratory Grenoble for their invaluable scientific input to the practical laboratory and analysis work, and to the writing and editing of the articles. Markos Mölsä from the Centre of Military Medicine is thanked for practical tips during the finalising steps of this thesis, Taru Koivulehto from Orion Diagnostica for tips and help with photos and Jennifer Rowland and Michael Hardman are thanked for the language revision of this thesis.

The path to this point has been a long one, and started already when I first got to work with biodefence issues. Therefore, I extend my sincerest thanks to Kari Kahiluoto from the Ministry for Foreign Affairs, for first taking me in to work in the BIOFIN project, and my previous director at THL, Olli Haikala from the Ministry of Social Affairs and Health, for leading me to the world of BWC and the negotiations on the verification protocol in the Geneva, and to the WMD inspection work with UNMOVIC. These activities led me to the most spectacular and memorable new adventures and courses around the globe together with colleagues from all UN member countries. Marja-Leena Vuorenpää is most warmely thanked for all the professional advice as well as the marvellous weekend trips to the Alps during the heavy negotiation weeks in Geneva. Ambassador Markku Reimaa is especially thanked for the refresments brought to us in the last hours of the negotiatiations in 2001 that were so highly admired by the other delegates as well. I thank Tarmo Humppi from the FDRA for the very first introduction to the world of biological agents and biodefence during my Masters studies. Without you, I would not have found this path.

Orion Diagnostica is thanked for granting me study leave for the time to write this theses and the Education Fund for financial support during this time. The Association for Protection,

Rescue, Security and Safety is thanked for financial support for the proofreading of the manuscript. The Doctoral Programme in Biomedicine (DPBM) of the University of Helsinki is thanked for support of my doctoral studies in the last few years.

I cannot go without thanking my best study friend, Anu Nurkka, the mother of my godson, for the happy companionship during so many laboratory courses. Annika Antonsson is thanked for splended time together in the microbiology courses at the University College Cork in Ireland during our Erasmus year. All my friends are thanked for so many happy moments spent in hobbies, travelling, sharing good food, and trekking in nature. You have made my life rich and cheered me up during difficult times. Thank you for being there for me.

Last, but certainly not the least, my deepest and heartfelt gratitude and thanks to my dearest family, my mother Anneli, my father Jouko and my big sister Tarja, for all their love, encouragement and support during all these years. I am forever grateful to have such an amazing and supportive family by my side. My furry friend, Josefina, thank you for keeping me company during all the long nightly hours writing this thesis, your warm loving purr has made it so much easier to keep going.

The most of all, I'm thankful for life, for all the amazing moments, for the blue sky and butterflies, crispy winter days and dark November evenings, and for every breath of air.

Vantaa, March 2017

Pirjo Matero

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